

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>6</sup>:</b> <b>C11D 3/386, A61K 7/48, C12N 15/57</b>		<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 95/10591</b> <b>(43) International Publication Date:</b> 20 April 1995 (20.04.95)
<b>(21) International Application Number:</b> PCT/US94/11776 <b>(22) International Filing Date:</b> 13 October 1994 (13.10.94)			<b>(74) Agents:</b> REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217 (US).  <b>(81) Designated States:</b> AM, AU, BB, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LK, LR, LT, LV, MD, MG, MN, NO, NZ, PL, RO, RU, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).
<b>(30) Priority Data:</b> 08/136,797 14 October 1993 (14.10.93) US 08/237,938 2 May 1994 (02.05.94) US  <b>(71) Applicant:</b> THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).  <b>(72) Inventors:</b> BAECK, André; 273, Putsesteenweg, B-2820 Bonheiden (BE). GHOSH, Chanchal, Kumar; 7005 Pine Mille Drive, West Chester, OH 45069 (US). GRAYCAR, Thomas, Paul; 1166 Manzanita, Pacifico, CA 94044 (US). BOTT, Richard, Ray; 3032 Hillside Drive, Burlingame, CA 94010 (US). WILSON, Lori, Jean; 33 El Bonito Way, Millbrae, CA 94030 (US). BRODE, Philip, Frederick, III; 5780 Squirrelsnest Lane, Cincinnati, OH 45252 (US). BARNETT, Bobby, Lee; 12175 Elkwood Drive, Cincinnati, OH 45240 (US). RUBINGH, Donn, Nelton; 8224 Sheed Road, Cincinnati, OH 45247 (US).			
<b>(54) Title:</b> PROTEASE-CONTAINING CLEANING COMPOSITIONS			
<b>(57) Abstract</b> <p>The present invention relates to cleaning compositions comprising protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions correspond to naturally-occurring subtilisin from <i>Bacillus amyloliquefaciens</i> or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as <i>Bacillus lentus</i> subtilisin).</p>			

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	GB	United Kingdom	MR	Mauritania
AU	Australia	GE	Georgia	MW	Malawi
BB	Barbados	GN	Guinea	NE	Niger
BE	Belgium	GR	Greece	NL	Netherlands
BF	Burkina Faso	HU	Hungary	NO	Norway
BG	Bulgaria	IE	Ireland	NZ	New Zealand
BJ	Benin	IT	Italy	PL	Poland
BR	Brazil	JP	Japan	PT	Portugal
BY	Belarus	KE	Kenya	RO	Romania
CA	Canada	KG	Kyrgyzstan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LU	Luxembourg	TD	Chad
CS	Czechoslovakia	LV	Latvia	TG	Togo
CZ	Czech Republic	MC	Monaco	TJ	Tajikistan
DE	Germany	MD	Republic of Moldova	TT	Trinidad and Tobago
DK	Denmark	MG	Madagascar	UA	Ukraine
ES	Spain	ML	Mali	US	United States of America
FI	Finland	MN	Mongolia	UZ	Uzbekistan
FR	France			VN	Viet Nam
GA	Gabon				

## PROTEASE-CONTAINING CLEANING COMPOSITIONS

5

10

This application is a continuation-in-part application of U.S. Application  
15 Serial No. 08/136,797, filed October 14, 1993 and U.S. Application Serial No.  
08/237,938, filed May 2, 1994, both incorporated herein by reference in their  
entirety.

TECHNICAL FIELD

The present invention relates to a variety of cleaning compositions  
20 comprising novel protease enzymes which are carbonyl hydrolase variants.

BACKGROUND

Enzymes make up the largest class of naturally occurring proteins.  
Each class of enzyme generally catalyzes (accelerates a reaction without being  
consumed) a different kind of chemical reaction. One class of enzymes, known  
25 as proteases, are known for their ability to hydrolyze (break down a compound  
into two or more simpler compounds with the uptake of the H and OH parts of a  
water molecule on either side of the chemical bond cleaved) other proteins.  
This ability to hydrolyze proteins has been taken advantage of by incorporating  
naturally occurring and protein engineered proteases as an additive to laundry  
30 detergent preparations. Many stains on clothes are proteinaceous and wide-  
specificity proteases can substantially improve removal of such stains.

Unfortunately, the efficacy level of these proteins in their natural,  
bacterial environment, frequently does not translate into the relatively unnatural  
wash environment. Specifically, protease characteristics such as thermal  
35 stability, pH stability, oxidative stability and substrate specificity are not  
necessarily optimized for utilization outside the natural environment of the  
enzyme.

The amino acid sequence of the protease enzyme determines the characteristics of the protease. A change of the amino acid sequence of the protease may alter the properties of the enzyme to varying degrees, or may even inactivate the enzyme, depending upon the location, nature and/or magnitude of the change in the amino acid sequence. Several approaches have been taken to alter the amino acid sequence of proteases in an attempt to improve their properties, with the goal of increasing the efficacy of the protease for cleaning uses such as in the wash environment. These approaches include altering the amino acid sequence to enhance thermal stability and to improve oxidation stability under quite diverse conditions.

Despite the variety of approaches described in the art, there is a continuing need for new effective variants of proteases useful for cleaning a variety of surfaces. It is therefore an object of the present invention to provide cleaning compositions containing protease enzymes which are carbonyl hydrolase variants having improved proteolytic activity, substrate specificity, stability and/or enhanced performance characteristics. These and other objects will become readily apparent from the detailed description which follows.

#### SUMMARY OF THE INVENTION

The present invention relates to cleaning compositions comprising:

(a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids, wherein said plurality of amino acid residues replaced in the precursor enzyme correspond to position +76 in combination with one or more of the following residues: +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered positions corresponds to naturally-occurring subtilisin from Bacillus amyloliquefaciens or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins (such as Bacillus lentus subtilisin); and

(b) one or more cleaning composition materials compatible with the protease enzyme.

The present invention also relates to methods for cleaning items in need of cleaning by contacting said item with a protease enzyme which is a carbonyl hydrolase variant as described herein. The invention therefore encompasses a method for cleaning fabrics comprising contacting, preferably with agitation,



said fabrics with an aqueous liquor containing said protease nzyme. The method can be carried out at temperatures below about 60°C but, of course, is quite effective at laundry temperatures up to the boil. The present invention also relates to a method for cleaning dishes by contacting a dish in need of cleaning with a protease enzyme as described herein. The present invention methods also include methods for personal cleansing , said methods comprising contacting the part of the human or lower animal body in need of cleaning with a protease enzyme as described herein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

10 Figs. 1 A-C depict the DNA and amino acid sequence for *Bacillus amyloliquefaciens* subtilisin and a partial restriction map of this gene (Seq. ID No.6).

Fig. 2 depicts the conserved amino acid residues among subtilisins from *Bacillus amyloliquefaciens* (BPN)' and *Bacillus lentus* (wild-type).

15 Figs. 3A and 3B depict the amino acid sequence of four subtilisins. The top line represents the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens* subtilisin (also sometimes referred to as subtilisin BPN') (Seq. ID No.7). The second line depicts the amino acid sequence of subtilisin from *Bacillus subtilis* (Seq. ID No.8). The third line depicts the amino acid sequence of subtilisin from *B. licheniformis* (Seq. ID No.9). The fourth line depicts the amino acid sequence of subtilisin from *Bacillus lentus* (also referred to as subtilisin 309 in PCT WO89/06276) (Seq. ID No.10). The symbol \* denotes the absence of specific amino acid residues as compared to subtilisin BPN'.

25 Fig. 4 depicts the construction of plasmid GGA274.

Fig. 5 depicts the construction of GGT274 which is an intermediate to certain expression plasmids used in this application.

30 Figs. 6A and 6B depict the DNA and amino acid sequence of subtilisin from *Bacillus lentus* (Seq. ID No.11). The mature subtilisin protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

Figs. 7A and 7B depict the DNA and amino acid sequence of a preferred embodiment of the invention (N76D/S103A/V104I) (Seq. ID No.12). The DNA in this figure has been modified by the methods described to encode aspartate at position 76, alanine at position 103 and isoleucine at position 104. The mature subtilisin variant protein is coded by the codons beginning at the codon GCG (334-336) corresponding to Ala.

35 Fig. 8 depicts the construction of vector pBCDAICAT.

Fig. 9 depicts the construction of vector pUCCATFNA.

Fig. 10 shows the stability of a preferred mutant enzyme compared to wild-type, in a liquid detergent formulation.

### DETAILED DESCRIPTION OF THE INVENTION

#### 5 1. Protease Enzymes:

The invention includes protease enzymes which are non-naturally-occurring carbonyl hydrolase variants having a different proteolytic activity, stability, substrate specificity, pH profile and/or performance characteristic as compared to the precursor carbonyl hydrolase from which the amino acid sequence of the variant is derived. The precursor carbonyl hydrolase may be a naturally-occurring carbonyl hydrolase or recombinant hydrolase. Specifically, such carbonyl hydrolase variants have an amino acid sequence not found in nature, which is derived by replacement of a plurality of amino acid residues of a precursor carbonyl hydrolase with different amino acids. The plurality of amino acid residues of the precursor enzyme correspond to position +76 in combination with one or more of the following residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274, where the numbered position corresponds to naturally-occurring subtilisin from *Bacillus amyloliquefaciens* or to equivalent amino acid residues in other carbonyl hydrolases or subtilisins, such as *Bacillus lentus* subtilisin.

The carbonyl hydrolase variants which are protease enzyme useful in the present invention compositions comprise replacement of amino acid residue +76 in combination with one or more additional modifications.

25 Preferably the variant protease enzymes useful for the present invention comprise the substitution, deletion or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 30 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably 35 the variant enzymes useful for the present invention comprise the substitution, deletion or insertion of an amino acid residue in the following combination of

residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104 of *B. amyloliquefaciens* subtilisin.

Variant DNA sequences encoding such carbonyl hydrolase or subtilisin variants are derived from a precursor DNA sequence which encodes a naturally-occurring or recombinant precursor enzyme. The variant DNA sequences are derived by modifying the precursor DNA sequence to encode the substitution of one or more specific amino acid residues encoded by the precursor DNA sequence corresponding to positions 76, 99, 101, 103, 104, 107, 123, 27, 105, 109, 126, 128, 135, 156, 166, 195, 197, 204, 206, 210, 216, 217, 218, 222, 260, 265 and/or 274, in *Bacillus amyloliquefaciens* or any combination thereof. Although the amino acid residues identified for modification herein are identified according to the numbering applicable to *B. amyloliquefaciens* (which has become the conventional method for identifying residue positions in all subtilisins), the preferred precursor DNA sequence useful for the present invention is the DNA sequence of *Bacillus lentus* as shown in Fig. 6 (Seq. ID No. 11).

These variant DNA sequences encode the insertion or substitution of the amino acid residue 76 in combination with one or more additional modification. Preferably the variant DNA sequences encode the substitution or insertion of amino acid residues in the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104; 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222. Most preferably the variant DNA sequences encode for the modification of the following combinations of residues: 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These recombinant DNA sequences encode carbonyl hydrolase variants having a novel amino acid sequence and, in general, at least one property which is substantially different from the same property of the enzyme encoded by the precursor carbonyl hydrolase DNA sequence. Such properties include proteolytic activity, substrate specificity, stability, altered pH profile and/or enhanced performance characteristics.

The protease enzymes useful herein encompass the substitution of any of the nineteen naturally occurring L-amino acids at the designated amino acid residue positions. Such substitutions can be made in any precursor subtilisin (procaryotic, eucaryotic, mammalian, etc.). Throughout this application reference is made to various amino acids by way of common one- and three-letter codes. Such codes are identified in Dale, J.W. (1989), Molecular Genetics of Bacteria, John Wiley & Sons, Ltd., Appendix B.

Preferably, the substitution to be made at each of the identified amino acid residue positions include but are not limited to: substitutions at position 76 including D, H, E, G, F, K, P and N; substitutions at position 99 including D, T, N, Q, G and S; substitutions at position 101 including G, D, K, L, A, E, S and R; substitutions at position 103 including Q, T, D, E, Y, K, G, R, S, and A; substitutions at position 104 including all nineteen naturally-occurring amino acids; substitutions at position 107 including V, L, M, Y, G, E, F, T, S, A, N and I; substitutions at position 123 including N, T, I, G, A, C, and S; substitutions at position 27 including K, N, C, V and T; substitutions at position 105 including A, D, G, R and N; substitutions at position 107 including A, L, V, Y, G, F, T, S and A; substitutions at position 109 including S, K, R, A, N and D; substitutions at position 126 including A, F, I, V and G; substitutions at position 128 including G, L and A; substitutions at position 135 including A, F, I, S and V; substitutions at position 156 including D, E, A, G, Q and K; substitutions at position 166 including all nineteen naturally-occurring amino acids; substitutions at position 195 including E; substitutions at position 197 including E; substitutions at position 204 including A, G, C, S and D; substitutions at position 206 including L, Y, N, D and E; substitutions at position 210 including L, I, S, C and F; substitutions at position 216 including V, E, T and K; substitutions at position 217 including all nineteen naturally-occurring amino acids; substitutions at position 218 including S, A, G, T and V; substitutions at position 222 including all nineteen naturally-occurring amino acids; substitutions at position 260 including P, N, G, A, S, C, K and D; substitutions at position 265 including N, G, A, S, C, K, Y and H; and substitutions at position 274 including A and S. The specifically preferred amino acid(s) to be substituted at each such position are designated below in Table I. Although specific amino acids are shown in Table I, it should be understood that any amino acid may be substituted at the identified residues.

Table I

Amino Acid

Preferred Amino Acid to

	<u>Residue</u>	<u>be Substituted/Inserted</u>
	+76	D,H
	+99	D,T,N,G
	+101	R,G,D,K,L,A,E
5	+103	A,Q,T,D,E,Y,K,G,R
	+104	I,Y,S,L,A,T,G,F,M,W,D,V,N
	+107	V,L,Y,G,F,T,S,A,N
	+123	S,T,I
	+27	K
10	+105	A,D
	+109	S,K,R
	+126	A,I,V,F
	+128	G,L
	+135	I,A,S
15	+156	E,D,Q
	+166	D,G,E,K,N,A,F,I,V,L
	+195	E
	+197	E
	+204	A,G,C
20	+206	L
	+210	I,S,C
	+216	V
	+217	H,I,Y,C,A,G,F,S,N,E,K
	+218	S
25	+222	A,Q,S,C,I,K
	+260	P,A,S,N,G
	+265	N,A,G,S
	+274	A,S

These protease enzymes containing *in vitro* mutations in *B. lentus* subtilisin at an amino acid residue equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin produces subtilisin variants exhibiting altered stability (e.g., modified autoproteolytic stability) over precursor subtilisins. (See Tables IV and VI.)

Also, *in vitro* mutation at residues equivalent to +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin, alone or in combination with each other

and in any combination with +76 mutations, produce subtilisin variants exhibiting altered proteolytic activity, altered thermal stability, altered pH profile, altered substrate specificity and/or altered performance characteristics.

5 Carbonyl hydrolases are protease enzymes which hydrolyze compounds containing



10 bonds in which X is oxygen or nitrogen. They include naturally-occurring carbonyl hydrolases and recombinant carbonyl hydrolases. Naturally-occurring carbonyl hydrolases principally include hydrolases, e.g., peptide hydrolases such as subtilisins or metalloproteases. Peptide hydrolases include  $\alpha$ -aminoacylpeptide hydrolase, peptidylamino acid hydrolase, acylamino hydrolase, serine carboxypeptidase, metallocarboxypeptidase, thiol proteinase, 15 carboxylproteinase and metalloproteinase. Serine, metallo, thiol and acid proteases are included, as well as endo and exo-proteases.

"Recombinant carbonyl hydrolase" refers to a carbonyl hydrolase in which the DNA sequence encoding the naturally-occurring carbonyl hydrolase is modified to produce a mutant DNA sequence which encodes the substitution, 20 insertion or deletion of one or more amino acids in the carbonyl hydrolase amino acid sequence. Suitable modification methods are disclosed herein, and in U.S. Patent 4,760,025 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258, the disclosure of which are incorporated herein by reference.

Subtilisins are bacterial or fungal carbonyl hydrolases which generally 25 act to cleave peptide bonds of proteins or peptides. As used herein, "subtilisin" means a naturally-occurring subtilisin or a recombinant subtilisin. A series of naturally-occurring subtilisins is known to be produced and often secreted by various microbial species. Amino acid sequences of the members of this series are not entirely homologous. However, the subtilisins in this series exhibit the 30 same or similar type of proteolytic activity. This class of serine proteases shares a common amino acid sequence defining a catalytic triad which distinguishes them from the chymotrypsin related class of serine proteases. The subtilisins and chymotrypsin related serine proteases both have a catalytic triad comprising aspartate, histidine and serine. In the subtilisin related 35 proteases the relative order of these amino acids, reading from the amino to carboxy terminus, is aspartate-histidine-serine. In the chymotrypsin related proteases the relative order, however, is histidine-aspartate-serine. Thus,

subtilisin herein refers to a serine protease having the catalytic triad of subtilisin related proteases. Examples include but are not limited to the subtilisins identified in Fig. 3 herein.

"Recombinant subtilisin" refers to a subtilisin in which the DNA  
5 sequence encoding the subtilisin is modified to produce a variant (or mutant) DNA sequence which encodes the substitution, deletion or insertion of one or more amino acids in the naturally-occurring subtilisin amino acid sequence. Suitable methods to produce such modification, and which may be combined with those disclosed herein, include those disclosed in U.S. Patent 4,760,025  
10 (RE 34,606), U.S. Patent 5,204,015 and U.S. Patent 5,185,258.

"Non-human carbonyl hydrolases" and the DNA encoding them may be obtained from many procaryotic and eucaryotic organisms. Suitable examples of procaryotic organisms include gram negative organisms such as *E. coli* or *Pseudomonas* and gram positive bacteria such as *Micrococcus* or *Bacillus*.  
15 Examples of eucaryotic organisms from which carbonyl hydrolase and their genes may be obtained include yeast such as *Saccharomyces cerevisiae*, fungi such as *Aspergillus* sp. and non-human mammalian sources such as, for example, *bovine* sp. from which the gene encoding the carbonyl hydrolase chymosin can be obtained. As with subtilisins, a series of carbonyl hydrolases  
20 can be obtained from various related species which have amino acid sequences which are not entirely homologous between the members of that series but which nevertheless exhibit the same or similar type of biological activity. Thus, non-human carbonyl hydrolase as used herein has a functional definition which refers to carbonyl hydrolases which are associated, directly or  
25 indirectly, with procaryotic and eucaryotic sources.

A "carbonyl hydrolase variant" has an amino acid sequence which is derived from the amino acid sequence of a "precursor carbonyl hydrolase." The precursor carbonyl hydrolases (such as a subtilisin) include naturally-occurring carbonyl hydrolases (subtilisin) and recombinant carbonyl hydrolases  
30 (subtilisin). The amino acid sequence of the carbonyl hydrolase variant is "derived" from the precursor hydrolase amino acid sequence by the substitution, deletion or insertion of one or more amino acids of the precursor amino acid sequence. Such modification is of the "precursor DNA sequence" which encodes the amino acid sequence of the precursor carbonyl hydrolase  
35 (subtilisin) rather than manipulation of the precursor carbonyl hydrolase (subtilisin) enzyme *per se*. Suitable methods for such manipulation of the precursor DNA sequence include methods disclosed herein, as well as

methods known to those skilled in the art (see, for example, EP 0 328299, WO89/06279 and the US patents and applications already referenced herein).

Specific residues corresponding to position +76 in combination with one or more of the following positions +99, +101, +103, +104, +107, +123, +27,  
5 +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 of *Bacillus amyloliquefaciens* subtilisin are identified herein for mutation. Preferably the modified residues are selected from the following combinations: 76/99; 76/101; 76/103; 76/104; 76/107; 76/123; 76/99/101; 76/99/103; 76/99/104; 76/101/103; 76/101/104;  
10 76/103/104; 76/104/107; 76/104/123; 76/107/123; 76/99/101/103; 76/99/101/104; 76/99/103/104; 76/101/103/104; 76/103/104/123; 76/104/107/123; 76/99/101/103/104; 76/99/103/104/123; 76/99/101/103/104/123; 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126;  
15 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222; and most preferably are 76/99; 76/104; 76/99/104; 76/103/104; 76/104/107; 76/101/103/104; 76/99/101/103/104 and 76/101/104. These amino acid position numbers refer to those assigned to the mature *Bacillus amyloliquefaciens* subtilisin sequence presented in Fig. 1. The protease  
20 enzymes useful in the present invention, however, are not limited to the mutation of this particular subtilisin but extends to precursor carbonyl hydrolases containing amino acid residues at positions which are "equivalent" to the particular identified residues in *Bacillus amyloliquefaciens* subtilisin. Preferably, the precursor subtilisin is *Bacillus lentus* subtilisin and the  
25 substitutions, deletions or insertions are made at the equivalent amino acid residue in *B. lentus* corresponding to those listed above.

A residue (amino acid) of a precursor carbonyl hydrolase is equivalent to a residue of *Bacillus amyloliquefaciens* subtilisin if it is either homologous (i.e., corresponding in position in either primary or tertiary structure) or analogous to  
30 a specific residue or portion of that residue in *Bacillus amyloliquefaciens* subtilisin (i.e., having the same or similar functional capacity to combine, react, or interact chemically).

In order to establish homology to primary structure, the amino acid sequence of a precursor carbonyl hydrolase is directly compared to the *Bacillus amyloliquefaciens* subtilisin primary sequence and particularly to a set of  
35 residues known to be invariant in subtilisins for which sequence is known. Fig. 2 herein shows the conserved residues as between *amyloliquefaciens* subtilisin



and *B. lentus* subtilisin. After aligning the conserved residues, allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of  
5 *Bacillus amyloliquefaciens* subtilisin are defined. Alignment of conserved  
residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Conservation of the catalytic triad, Asp32/His64/Ser221 should be maintained.

10 For example, in Fig. 3 the amino acid sequence of subtilisin from *Bacillus amyloliquefaciens*, *Bacillus subtilis*, *Bacillus licheniformis* (*carlsbergensis*) and *Bacillus lentus* are aligned to provide the maximum amount of homology between amino acid sequences. A comparison of these sequences shows that there are a number of conserved residues contained in  
15 each sequence. These conserved residues (as between BPN' and *B. lentus*) are identified in Fig. 2.

These conserved residues, thus, may be used to define the corresponding equivalent amino acid residues of *Bacillus amyloliquefaciens* subtilisin in other carbonyl hydrolases such as subtilisin from *Bacillus lentus*  
20 (PCT Publication No. W089/06279 published July 13, 1989), the preferred subtilisin precursor enzyme herein, or the subtilisin referred to as PB92 (EP O 328 299), which is highly homologous to the preferred *Bacillus lentus* subtilisin. The amino acid sequences of certain of these subtilisins are aligned in Figs. 3A and 3B with the sequence of *Bacillus amyloliquefaciens* subtilisin to produce  
25 the maximum homology of conserved residues. As can be seen, there are a number of deletions in the sequence of *Bacillus lentus* as compared to *Bacillus amyloliquefaciens* subtilisin. Thus, for example, the equivalent amino acid for Val165 in *Bacillus amyloliquefaciens* subtilisin in the other subtilisins is isoleucine for *B. lentus* and *B. licheniformis*.

30 Thus, for example, the amino acid at position +76 is asparagine (N) in both *B. amyloliquefaciens* and *B. lentus* subtilisins. In the preferred subtilisin variant useful in the invention, however, the amino acid equivalent to +76 in *Bacillus amyloliquefaciens* subtilisin is substituted with aspartate (D). A comparison of all the amino acid residues identified herein for substitution  
35 versus the preferred substitution for each such position is provided in Table II for illustrative purposes.

Table II

	<u>+76</u>	<u>+99</u>	<u>+101</u>	<u>+103</u>	<u>+104</u>	<u>+107</u>	<u>+123</u>
<i>B. amyloliquefaciens</i> (wild-type)	N	D	S	Q	Y	I	N

5

<i>B. lentus</i> (wild-type)	N	S	S	S	V	I	N
------------------------------	---	---	---	---	---	---	---

Most Preferred Substitution D D R A I/Y V S

- Equivalent residues may also be defined by determining homology at the level of tertiary structure for a precursor carbonyl hydrolase whose tertiary structure has been determined by x-ray crystallography. Equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the precursor carbonyl hydrolase and *Bacillus amyloliquefaciens* subtilisin (N on N, CA on CA, C on C and O on O) are within 0.13nm and preferably 0.1nm after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the carbonyl hydrolase in question to the *Bacillus amyloliquefaciens* subtilisin. The best model is the crystallographic model giving the lowest R factor for experimental diffraction data at the highest resolution available.

$$R \text{ factor} = \frac{\sum_h |F_o(h)| - |F_c(h)|}{\sum_h |F_o(h)|}$$

- Equivalent residues which are functionally analogous to a specific residue of *Bacillus amyloliquefaciens* subtilisin are defined as those amino acids of the precursor carbonyl hydrolases which may adopt a conformation such that they either alter, modify or contribute to protein structure, substrate binding or catalysis in a manner defined and attributed to a specific residue of the *Bacillus amyloliquefaciens* subtilisin. Further, they are those residues of the precursor carbonyl hydrolase (for which a tertiary structure has been obtained by x-ray crystallography) which occupy an analogous position to the extent that, although the main chain atoms of the given residue may not satisfy the criteria of equivalence on the basis of occupying a homologous position, the atomic coordinates of at least two of the side chain atoms of the residue lie within 0.13nm of the corresponding side chain atoms of *Bacillus amyloliquefaciens* subtilisin. The coordinates of the three dimensional structure of *Bacillus amyloliquefaciens* subtilisin are set forth in EPO Publication No. 0 251 446 (equivalent to U.S. Patent Application SN

08/212,291, the disclosure of which is incorporated herein by reference) and can be used as outlined above to determine equivalent residues on the level of tertiary structure.

Some of the residues identified for substitution, insertion or deletion are conserved residues whereas others are not. In the case of residues which are not conserved, the replacement of one or more amino acids is limited to substitutions which produce a variant which has an amino acid sequence that does not correspond to one found in nature. In the case of conserved residues, such replacements should not result in a naturally-occurring sequence. The carbonyl hydrolase variants useful in the present invention include the mature forms of carbonyl hydrolase variants, as well as the pro- and prepro-forms of such hydrolase variants. The prepro-forms are the preferred construction since this facilitates the expression, secretion and maturation of the carbonyl hydrolase variants.

"Prosequence" refers to a sequence of amino acids bound to the N-terminal portion of the mature form of a carbonyl hydrolase which when removed results in the appearance of the "mature" form of the carbonyl hydrolase. Many proteolytic enzymes are found in nature as translational proenzyme products and, in the absence of post-translational processing, are expressed in this fashion. A preferred prosequence for producing carbonyl hydrolase variants, specifically subtilisin variants, is the putative prosequence of *Bacillus amyloliquefaciens* subtilisin, although other subtilisin prosequences may be used. In the Examples, the putative prosequence from the subtilisin from *Bacillus lentus* (ATCC 21536) is used.

A "signal sequence" or "presequence" refers to any sequence of amino acids bound to the N-terminal portion of a carbonyl hydrolase or to the N-terminal portion of a prohydrolase which may participate in the secretion of the mature or pro forms of the hydrolase. This definition of signal sequence is a functional one, meant to include all those amino acid sequences encoded by the N-terminal portion of the subtilisin gene or other secretable carbonyl hydrolases which participate in the effectuation of the secretion of subtilisin or other carbonyl hydrolases under native conditions. The protease enzymes useful for the present invention utilize such sequences to effect the secretion of the carbonyl hydrolase variants as described herein. A preferred signal sequence used in the Examples comprises the first seven amino acid residues of the signal sequence from *Bacillus subtilis* subtilisin fused to the remainder of the signal sequence of the subtilisin from *Bacillus lentus* (ATCC 21536).

A "prepro" form of a carbonyl hydrolase variant consists of the mature form of the hydrolase having a prosequence operably linked to the amino terminus of the hydrolase and a "pre" or "signal" sequence operably linked to the amino terminus of the prosequence.

- 5 "Expression vector" refers to a DNA construct containing a DNA  
sequence which is operably linked to a suitable control sequence capable of  
effecting the expression of said DNA in a suitable host. Such control  
sequences include a promoter to effect transcription, an optional operator  
sequence to control such transcription, a sequence encoding suitable mRNA  
10 ribosome binding sites and sequences which control termination of  
transcription and translation. The vector may be a plasmid, a phage particle, or  
simply a potential genomic insert. Once transformed into a suitable host, the  
vector may replicate and function independently of the host genome, or may, in  
some instances, integrate into the genome itself. In the present specification,  
15 "plasmid" and "vector" are sometimes used interchangeably as the plasmid is  
the most commonly used form of vector at present. However, included herein  
are such other forms of expression vectors which serve equivalent functions  
and which are, or become, known in the art.

- The "host cells" used in the present invention generally are procaryotic  
20 or eucaryotic hosts which preferably have been manipulated by the methods  
disclosed in U.S. Patent 4,760,025 (RE 34,606) to render them incapable of  
secreting enzymatically active endoprotease. A preferred host cell for  
expressing subtilisin is the *Bacillus* strain BG2036 which is deficient in  
enzymatically active neutral protease and alkaline protease (subtilisin). The  
25 construction of strain BG2036 is described in detail in U.S. Patent 5,264,366.  
Other host cells for expressing subtilisin include *Bacillus subtilis* 1168 (also  
described in U.S. Patent 4,760,025 (RE 34,606) and U.S. Patent 5,264,366, the  
disclosure of which are incorporated herein by reference), as well as any  
suitable *Bacillus* strain such as *B. licheniformis*, *B. lentus*, etc.

- 30 Host cells are transformed or transfected with vectors constructed using  
recombinant DNA techniques. Such transformed host cells are capable of  
either replicating vectors encoding the carbonyl hydrolase variants or  
expressing the desired carbonyl hydrolase variant. In the case of vectors  
which encode the pre- or prepro-form of the carbonyl hydrolase variant, such  
35 variants, when expressed, are typically secreted from the host cell into the host  
cell medium.

"Operably linked," when describing the relationship between two DNA regions, simply means that they are functionally related to each other. For example, a presequence is operably linked to a peptide if it functions as a signal sequence, participating in the secretion of the mature form of the protein most probably involving cleavage of the signal sequence. A promoter is operably linked to a coding sequence if it controls the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The genes encoding the naturally-occurring precursor carbonyl hydrolase may be obtained in accord with the general methods known to those skilled in the art. The methods generally comprise synthesizing labeled probes having putative sequences encoding regions of the hydrolase of interest, preparing genomic libraries from organisms expressing the hydrolase, and screening the libraries for the gene of interest by hybridization to the probes. Positively hybridizing clones are then mapped and sequenced. The *B. lentus* gene used in the Examples is cloned as described in Example 1 of U.S. Patent 5,185,258, the disclosure of which is incorporated herein. The BPN' gene used in the Examples is cloned as described in Example 1 in RE 34,606, the disclosure of which is incorporated herein.

The cloned carbonyl hydrolase is then used to transform a host cell in order to express the hydrolase. The hydrolase gene is then ligated into a high copy number plasmid. This plasmid replicates in hosts in the sense that it contains the well-known elements necessary for plasmid replication: a promoter operably linked to the gene in question (which may be supplied as the gene's own homologous promoter if it is recognized, i.e., transcribed, by the host), a transcription termination and polyadenylation region (necessary for stability of the mRNA transcribed by the host from the hydrolase gene in certain eucaryotic host cells) which is exogenous or is supplied by the endogenous terminator region of the hydrolase gene and, desirably, a selection gene such as an antibiotic resistance gene that enables continuous cultural maintenance of plasmid-infected host cells by growth in antibiotic-containing media. High copy number plasmids also contain an origin of replication for the host, thereby enabling large numbers of plasmids to be generated in the cytoplasm without chromosomal limitations. However, it is within the scope herein to integrate multiple copies of the hydrolase gene into host genome. This is facilitated by procaryotic and eucaryotic organisms which are particularly susceptible to homologous recombination.

The genes used in the present examples are a natural *B. lentus* gene and a natural *B. amyloliquefaciens* gene. Alternatively, a synthetic gene encoding a naturally-occurring or mutant precursor carbonyl hydrolase (subtilisin) may be produced. In such an approach, the DNA and/or amino acid sequence of the precursor hydrolase (subtilisin) is determined. Multiple, overlapping synthetic single-stranded DNA fragments are thereafter synthesized, which upon hybridization and ligation produce a synthetic DNA encoding the precursor hydrolase. An example of synthetic gene construction is set forth in Example 3 of U.S. Patent 5,204,015, the disclosure of which is incorporated herein by reference.

Once the naturally-occurring or synthetic precursor carbonyl hydrolase gene has been cloned, a number of modifications are undertaken to enhance the use of the gene beyond synthesis of the naturally-occurring precursor carbonyl hydrolase. Such modifications include the production of recombinant carbonyl hydrolases as disclosed in U.S. Patent 4,760,025 (RE 34,606) and EPO Publication No. 0 251 446 and the production of carbonyl hydrolase variants described herein.

The following cassette mutagenesis method may be used to facilitate the construction and identification of the carbonyl hydrolase variants useful in the present invention, although other methods including site-directed mutagenesis may be used. First, the naturally-occurring gene encoding the hydrolase is obtained and sequenced in whole or in part. Then the sequence is scanned for a point at which it is desired to make a mutation (deletion, insertion or substitution) of one or more amino acids in the encoded enzyme. The sequences flanking this point are evaluated for the presence of restriction sites for replacing a short segment of the gene with an oligonucleotide pool which when expressed will encode various mutants. Such restriction sites are preferably unique sites within the hydrolase gene so as to facilitate the replacement of the gene segment. However, any convenient restriction site which is not overly redundant in the hydrolase gene may be used, provided the gene fragments generated by restriction digestion can be reassembled in proper sequence. If restriction sites are not present at locations within a convenient distance from the selected point (from 10 to 15 nucleotides), such sites are generated by substituting nucleotides in the gene in such a fashion that neither the reading frame nor the amino acids encoded are changed in the final construction. Mutation of the gene in order to change its sequence to conform to the desired sequence is accomplished by M13 primer extension in

accord with generally known methods. The task of locating suitable flanking regions and evaluating the needed changes to arrive at two convenient restriction site sequences is made routine by the redundancy of the genetic code, a restriction enzyme map of the gene and the large number of different restriction enzymes. Note that if a convenient flanking restriction site is available, the above method need be used only in connection with the flanking region which does not contain a site.

Once the naturally-occurring DNA or synthetic DNA is cloned, the restriction sites flanking the positions to be mutated are digested with the cognate restriction enzymes and a plurality of end termini-complementary oligonucleotide cassettes are ligated into the gene. The mutagenesis is simplified by this method because all of the oligonucleotides can be synthesized so as to have the same restriction sites, and no synthetic linkers are necessary to create the restriction sites.

As used herein, proteolytic activity is defined as the rate of hydrolysis of peptide bonds per milligram of active enzyme. Many well known procedures exist for measuring proteolytic activity (K. M. Kalisz, "Microbial Proteinases," Advances in Biochemical Engineering/Biotechnology, A. Fiechter ed., 1988). In addition to or as an alternative to modified proteolytic activity, the variant enzymes of the present invention may have other modified properties such as  $K_m$ ,  $K_{cat}$ ,  $K_{cat}/K_m$  ratio and/or modified substrate specificity and/or modified pH activity profile. These enzymes can be tailored for the particular substrate which is anticipated to be present, for example, for hydrolytic processes such as laundry uses.

One objective can be to secure a variant carbonyl hydrolase having altered proteolytic activity as compared to the precursor carbonyl hydrolase, since increasing such activity (numerically larger) enables the use of the enzyme to more efficiently act on a target substrate. Also of interest are variant enzymes having altered thermal stability and/or altered substrate specificity as compared to the precursor. Preferably the carbonyl hydrolase to be mutated is a subtilisin. Specific amino acids useful to obtain such results in subtilisin-type carbonyl hydrolases at residues equivalent to +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 or any combination thereof in *Bacillus amyloliquefaciens* subtilisin are presented in the Examples. In some instances, lower proteolytic activity may be desirable. Conversely, in some instances it may be desirable to increase the proteolytic

activity of the variant enzyme versus its precursor. Additionally, increases or decreases (alteration) of the stability of the variant, whether alkaline or thermal stability, may be desirable. Increases or decreases in  $K_{cat}$ ,  $K_m$  or  $K_{cat}/K_m$  are specific to the substrate used to determine these kinetic parameters.

5 Also, it has been determined that residues equivalent to +76 in combination with a number of other modifications in subtilisin are important in modulating overall stability and/or proteolytic activity of the enzyme. Thus, as set forth in the Examples, the Asparagine (N) in *Bacillus lentus* subtilisin at equivalent position +76 can be substituted with Aspartate (D) in the preferred  
10 protease enzymes in combination with modification of one or more of the following amino acid residues +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 to produce enhanced stability and/or enhanced activity of the resulting mutant enzyme.

15 The most preferred protease enzymes useful in this invention are set forth in the Examples. These include the following specific combinations of substituted residues: N76D/S99D; N76D/V104I; N76D/S99D/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V and N76D/S101R/S103A/V104I. These substitutions are preferably made in  
20 *Bacillus lentus* (recombinant or native-type) subtilisin, although the substitutions may be made in any *Bacillus* subtilisin.

Based on the results obtained with this and other variant subtilisins, it is apparent that residues in carbonyl hydrolases (preferably subtilisin) equivalent to positions +76, +99, +101, +103, +104, +107, +123, +27, +105, +109, +126,  
25 +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265 and/or +274 in *Bacillus amyloliquefaciens* are important to the proteolytic activity, performance and/or stability of these enzymes and the cleaning or wash performance of such variant enzymes.

The following is presented by way of example for manufacturing  
30 protease enzymes useful in the present invention compositions.

#### Protease Manufacture Example

##### Construction for the Expression of GG36 Gene in *B. subtilis*

The cloning and the construction for expression of the subtilisin gene from *B. lentus* is performed essentially the same as that described in U.S. Patent  
35 5,185,258. The plasmid GGA274 (described in Fig. 4 herein) is further modified in the following manner, as shown in Fig. 5. The PstI site that is introduced during the construction of the GGA274 plasmid is removed by the



oligonucleotide directed mutagenesis described below, with an oligonucleotide having the following sequence: 5' GAAGCTGCAACTCGTTAA 3' (Seq. ID No.1). The underlined "A" residue eliminates the recognition sequence of restriction enzyme PstI and changes the corresponding amino acid residue from alanine to threonine at position 274. Threonine at position 274 is the wild-type residue originally found in the cloned *B. lentus* subtilisin gene sequences. The DNA segment encoding subtilisin is excised from the plasmid GGA274 or its derivatives (GGT274 shown in Fig. 5) by EcoRI and BamHI digest. The DNA fragment is subcloned back into Bacteriophage M13-based vectors, such as MP19, for mutagenesis. After mutagenesis, the EcoRI and HindIII digest, followed by cloning, are performed to move the mutated subtilisin gene back into an expression plasmid like GGA274 for the expression and the recovery of mutated subtilisin proteins.

#### Oligonucleotide-Directed Mutagenesis

Oligonucleotide-directed mutagenesis is performed as described in Zoller, M. et al. (1983), Methods Enzymol., 100:468-500. As an example, a synthetic oligonucleotide of the sequence 5' GCTGCTCTAGACAATTCG 3' (Seq. ID No.2) is used to change the amino acid residue at position 76 from asparagine (N) to aspartic acid (D), or N76D. The underlined "G" and "C" residues denote changes from the wild-type gene sequence. The CA keeps the leucine at position +75 and changes the amino acid sequence to introduce an XbaI recognition site of the XbaI restriction enzyme (TCTAGA), while the change at GAC changes asparagine at +76 to aspartate.

For mutagenesis at positions 99, 101, 103 and 104, different oligonucleotides can be used depending on the combination of mutations desired. For example, an oligonucleotide of the sequence 5' GTATTAGGGGCGGACGGTCGAGGCGCCATCAGCTCGATT 3' (Seq. ID No.3) is used to simultaneously make the following changes: S99D; S101R; S103A and V104I in a single subtilisin molecule. Similarly, oligonucleotides of the sequence 5' TCAGGTTCCGTCTCGAGCGTTGCCCAAGGATTG 3' (Seq. ID No.4) and 5' CACGTTGCTAGCTTGAGTTTAG 3' (Seq. ID No.5) are utilized to generate I107V and N123S, respectively. Again, the underlined residues denote changes from wild-type sequences which produce desired changes either in amino acid sequences or restriction enzyme recognition sequences.

#### Proteolytic Activity of Subtilisin Variants

Following the methods of Oligonucleotide-Directed Mutagenesis hereinbefore, the variants listed in Table III are made. Proteolytic activity of

each of these subtilisin variants is shown in Table III. The kinetic parameters  $k_{cat}$ ,  $K_M$ , and  $k_{cat}/K_M$  are measured for hydrolysis of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide using the method described in P. Bonneau et al. (1991) J. Am. Chem. Soc., Vol. 113, No. 3, p.

- 5 1030. Briefly, a small aliquot of subtilisin variant stock solution is added to a 1 cm cuvette containing substrate dissolved in 0.1M Tris-HCL buffer, pH 8.6, and thermostated at 25°C. The reaction progress is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm. Kinetic parameters are obtained by using a non-linear
- 10 regression algorithm to fit the reaction velocity and product concentration for each reaction to the Michaelis-Menten equation.

Table III

Kinetic Parameters  $k_{cat}$ ,  $K_M$  and  $k_{cat}/K_M$   
Measured for Bacillus lentus Subtilisin and Variants

<u>Proteas #</u>	<u>Enzyme</u> <u>Variants</u>	<u><math>k_{cat}(s^{-1})</math></u>	<u><math>K_M(M)</math></u>	<u><math>k_{cat}/K_M</math></u> <u><math>(s^{-1}M^{-1})</math></u>
-	<u>B. lentus</u> Subtilisin	170	0.00078	$2.18 \times 10^5$
-	N76D	219	0.0008	$2.74 \times 10^5$
1	N76D/S99D	88	0.00061	$1.44 \times 10^5$
2	N76D/S101R	371	0.0013	$2.85 \times 10^5$
3	N76D/S103A	400	0.0014	$2.86 \times 10^5$
4	N76D/V104I	459	0.0011	$4.17 \times 10^5$
5	N76D/I107V	219	0.0011	$1.99 \times 10^5$
6	N76D/N123S	115	0.0018	$6.40 \times 10^4$
7	N76D/S99D/S101R	146	0.00038	$3.84 \times 10^5$
8	N76D/S99D/S103A	157	0.0012	$1.31 \times 10^5$
9	N76D/S99D/V104I	247	0.00097	$2.55 \times 10^5$
10	N76D/S101R/S103A	405	0.00069	$5.90 \times 10^5$
11	N76D/S101R/V104I	540	0.00049	$1.10 \times 10^6$
12	N76D/S103A/V104I	832	0.0016	$5.20 \times 10^5$
13	N76D/V104I/I107V	497	0.00045	$1.10 \times 10^6$
14	N76D/V104Y/I107V	330	0.00017	$1.90 \times 10^6$
15	N76D/V104I/N123S	251	0.0026	$9.65 \times 10^4$
16	N76D/I107V/N123S	147	0.0035	$4.20 \times 10^4$
17	N76D/S99D/S101R/S103A	242	0.00074	$3.27 \times 10^5$
18	N76D/S99D/S101R/V104I	403	0.00072	$5.60 \times 10^5$
19	N76D/S99D/S103A/V104I	420	0.0016	$2.62 \times 10^5$
20	N76D/S101R/S103A/V104I	731	0.00065	$1.12 \times 10^6$
21	N76D/S103A/V104I/N123S	321	0.0026	$1.23 \times 10^5$
22	N76D/V104I/I107V/N123S	231	0.003	$7.70 \times 10^4$
23	N76D/S99D/S101R/S103A/V104I	624	0.00098	$6.37 \times 10^5$
24	N76D/S99D/S103A/V104I/N123S	194	0.0043	$4.51 \times 10^4$
25	N76D/S99D/S101R/S103A/V104I/N123S	311	0.0023	$1.35 \times 10^5$

The results listed in Table III indicate that all of the subtilisin variants tested retain proteolytic activity. Further, detailed analysis of the data reveal that proteolytic activity is significantly altered for *Bacillus lentus* subtilisin by the various combinations of substitutions at amino acid residues equivalent to positions 76, 99, 101, 103, 104, 107 and 123 in *Bacillus amyloliquefaciens*.

Thermal Stability of Subtilisin Variants

- A comparison of thermal stability observed for *Bacillus lentus* subtilisin and the variants of the present invention made by the process of Oligonucleotide-Directed Mutagenesis hereinbefore is shown in Table IV. Purified enzyme, 15 ug/ml in 0.1 M glycine 0.01% Tween-80 pH 10.0, with or without 50 mM CaCl<sub>2</sub>, is aliquotted into small tubes and incubated at 10°C for 5 minutes, 10°C to 60°C over 1 minute, and 60°C for 20 minutes. Tubes are then placed on ice for 10 minutes. Aliquots from the tubes are assayed for enzyme activity by addition to 1 cm cuvettes containing 1.2 mM of the synthetic peptide substrate succinyl-L-ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time. Data are presented as percent activity prior to heating. The results listed in Table IV indicate that a vast majority of variants exhibit thermal stability comparable to *Bacillus lentus* subtilisin (24 out of 26) in the test condition with 50mM CaCl<sub>2</sub> added. In the test condition without 50mM CaCl<sub>2</sub> added, a vast majority of variants (19 out of 26) are significantly more stable than *Bacillus lentus* subtilisin. Further, the variants N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V and N76D/S101R/S103A/V104I are significantly more stable than the single substitution variant N76D in the test condition without 50mM CaCl<sub>2</sub> added.

Table IV

Thermal Stability Measured for *Bacillus lentus* Subtilisin and Variants  
At pH 10, 60°C, +/- 50mM CaCl<sub>2</sub> Added

Enzyme	% Initial Activity Remaining	
	-CaCl <sub>2</sub>	+CaCl <sub>2</sub>
<u>B. lentus</u> Subtilisin	2	96
N76D	34	97
N76D/S99D	49	98
N76D/S101R	0	82
N76D/S103A	26	92
N76D/V104I	58	98
N76D/I107V	32	96
N76D/N123S	0	97
N76D/S99D/S101R	30	100
N76D/S99D/S103A	36	100
N76D/S99D/V104I	48	97

N76D/S101R/S103A	26	100
N76D/S101R/V104I	38	100
N76D/S103A/V104I	58	100
N76D/V104I/I107V	60	97
N76D/V104Y/I107V	48	74
N76D/V104I/N123S	0	98
N76D/I107V/N123S	16	100
N76D/S99D/S101R/S103A	38	100
N76D/S99D/S101R/V104I	33	100
N76D/S99D/S103A/V104I	38	98
N76D/S101R/S103A/V104I	40	99
N76D/S103A/V104I/N123S	1	98
N76D/V104I/I107V/N123S	3	99
N76D/S99D/S101R/S103A/V104I	36	99
N76D/S99D/S103A/V104I/N123S	2	95
N76D/S99D/S101R/S103A/V104I/N123S	0	100

Oligonucleotide-Directed Mutagenesis with  
Single-Stranded DNA Template Generated from Phagemid

A. Construction of *B. lentus* Variants

- The mutagenesis protocol is essentially the same as described above in
- 5 Oligonucleotide-Directed Mutagenesis. The single-stranded DNA template is generated by phagemid method. To construct the phagemid vector for generating the single-stranded DNA template we first construct the vector pBCDAICAT. The flow chart of vector construction is outlined in Figure 8. First, the *C1aI* to *C1aI* fragment encoding the CAT gene from pC194 plasmid
  - 10 (Horinouchi, S. and Weisblum, B., J. Bacteriol., **150**:8-15, 1982) is cloned into the *AccI* site of polylinker region of pUC19 (New England BioLabs, Beverly, MA) to make plasmid pUCCHL and the *EcoRI*-*DraI* 0.6 KB fragment from the 5' end of the GG36DAI encoding DNA is cloned into the *EcoRI* and *EcoRV* sites of pBSKS plasmid (Stratagene, Inc., San Diego, CA) to make pBC2SK5. The
  - 15 single *EcoRI* site of the plasmid pBC2SK5 is eliminated by *EcoRI* digestion, followed by filling in catalyzed-by-T4 DNA polymerase, and religation to generate the plasmid pBC2SK-5R which does not have the *EcoRI* site. The *EcoRI*-*DraI* fragment which is cloned into pBCSK-5R is isolated as a *PstI*-*HindIII* fragment and cloned into the *PstI*-*HindIII* site of the pUCCHL (part of the
  - 20 polylinker of pUC19) to generate plasmid pUCCHL5R. The encoding sequence of GG36DAI gene is excised as an *EcoRI*-*BamHI* fragment and

cloned into the *EcoRI-BamHI* sites of pUCCHL5R to make pUCCAT. The large *EcoRI-HindIII* fragment of pUCCAT is then cloned into the *EcoRI* and *HindIII* sites of BS2KS+ to generate the plasmid pBCDAICAT.

To generate single-stranded DNA, *E. coli*-containing pBCDAICAT are infected with phage R408 (obtained from Stratagene, San Diego, CA) following the protocol described in Russel, M., Kidd, S. and Kelley, M.R., GENE 45:333-338, 1986. Once the single-stranded DNA template is available, standard mutagenesis methods as described above in Oligonucleotide-Directed Mutagenesis are carried out. The construction of certain mutants is detailed below for illustrative purposes.

For the construction of *B. lentus* (GG36) N76D/S103A/V104I/L217H, an *EcoRI-BamHI* DNA fragment encoding GG36 N76D/S103A/V104I is used in the construction of pUCCAT (see Fig. 8) to generate the plasmid pBCDAICAT. After the single-stranded DNA template is made following the protocol described above, a mutagenesis primer with the following sequence

\* \* \* \* \* x *C1aI*.

5' TAT GCC AGC CAC AAC GGT ACT TCG ATG GCT 3' (Seq. ID No.13)

is used to make the L217H. As before, the underlined residues denote the nucleotide changes that are made and the x *C1aI* indicates that the existing *C1aI* site is eliminated after the mutagenesis. The mutagenesis protocol is as described in Oligonucleotide-Directed Mutagenesis hereinbefore. After the mutagenesis, plasmid DNA is first screened for the elimination of the *C1aI* site and those clones missing the *C1aI* site are subjected to DNA sequence analysis to verify the DNA sequence which made the L to H change at the 217th amino acid residue.

#### B. Construction of BPN' Variants and their Expression in *B. subtilis*

The construction of *B. amyloliquefaciens* (BPN')

N76D/Q103A/Y104I/Y217L is done in a similar fashion except in two consecutive steps. N76D is first introduced into BPN' Y217L to make BPN' N76D/Y217L and a second mutagenesis is done to convert BPN' N76D/Y217L to BPN' N76D/Q103A/Y104I/Y217L. To generate the single-stranded DNA template for the first mutagenesis, an *EcoRI-BamHI* fragment encoding BPN' Y217L subtilisin (derived from the Y217L plasmid described in Wells, J., et al., PNAS, 84, 5167, 1087) is used to construct a plasmid pUCCATFNA (see Fig. 9). The pUCCATFNA plasmid containing BPN' Y217L is used to construct the pBCFNACAT plasmid (Fig. 9). Single-stranded DNA is generated as described above. To generate BPN' N76D/Y217L, an oligonucleotide primer with the sequence

\* \*\*\* \*\* *Xba*I

5' C ACA GTT GCG GCT CTA GAT AAC TCA ATC GGT G 3' (Seq. ID No.14)

- is used to generate the change N76D. Single-stranded DNA is then prepared from the pBCFNACAT plasmid containing the BPN' N76D/Y217L (the pBCFNACAT plasmid after N76D mutagenesis) and mutagenized with another oligonucleotide with the sequence

\* \*\*\* \*\* x *Pvu*II

- 5' GCT GAC GGT TCC GGC GCT ATT AGT TGG ATC ATT 3' (Seq. ID No.15)

- to obtain BPN' N76D/Q103A/Y104I/Y217L. All steps involved in the cloning, the single-stranded DNA preparation, the mutagenesis, and the screening for mutants are carried out as described above. Expression of the BPN' gene and its variants are achieved by integrating plasmid DNA (pBCFNACAT containing the different variants' BPN' gene) directly into a protease-deficient strain of *Bacillus subtilis* as described in RE 34,606.

- Numerous variants are made as per the teachings of these Protease Manufacture Examples. Kinetics data and stability data are generated for such variants. The kinetics data are generated using the methods described hereinbefore and are provided in Table V. The stability data are generated as detailed herein. Results are shown in Table VI.

#### Thermal Stability Assay Procedure

- Purified enzyme is buffer-exchanged into 0.1 M glycine pH 10.0, 0.01% Tween-80 by applying the enzyme to a column consisting of Sephadex G-25 equilibrated with this buffer and eluting the enzyme from the column using the same buffer.

To a tube containing 0.1 M glycine, 0.01% Tween-80 pH 10.0 thermostatted at 60°C, the buffer-exchanged enzyme is added to give a final enzyme concentration of 15 ug/ml.

- Aliquots are removed from the 60°C incubation at various times and immediately assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide dissolved in 0.1 M tris-HCL buffer, pH 8.6, thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410 nm as a function of time.

Half-life, which is the length of time required for 50% enzyme inactivation, is determined from the first-order plot of reaction velocity as a function of the time of incubation at 60°C.

The data are presented in Table VI as percent of the half-life determined for *Bacillus lentus* subtilisin (GG36) under identical conditions.

Table V

Enzyme	kcat (s <sup>-1</sup> )	KM (mM)	kcat/KM (s <sup>-1</sup> M <sup>-1</sup> )
B. lentus subtilisin	170	0.78	2.20E+05
N76D/S103G/V104I*	380	1.4	2.70E+05
N76D/S103A/V104F	730	0.33	2.20E+06
N76D/S103A/V104N	790	2.8	2.80E+05
N76D/S103A/V104S	170	0.83	2.00E+05
N76D/S103A/V104T	370	1.9	2.00E+05
N76D/S103A/V104W	880	0.31	2.80E+06
N76D/S103A/V104Y	690	0.5	1.40E+06
K27R/N76D/V104Y/N123S	500	1.2	4.20E+05
N76D/S101G/S103A/V104I*	620	1.3	4.80E+05
N76D/S103A/V104I/S105A*	550	1.3	4.20E+05
N76D/S103A/V104I/S105D*	440	1.7	2.60E+05
N76D/S103A/V104T/I107A*	120	5.7	2.10E+04
N76D/S103A/V104T/I107L*	310	3.2	9.70E+04
N76D/S103A/V104I/L126A	90	2.2	4.10E+04
N76D/S103A/V104I/L126F	180	1.9	9.50E+04
N76D/S103A/V104I/L126I	100	2.4	4.20E+04
N76D/S103A/V104I/L126V	64	3.2	2.00E+04
N76D/S103A/V104I/S128G*	560	1.7	3.30E+05
N76D/S103A/V104I/S128L*	430	3.8	1.10E+05
N76D/S103A/V104I/L135A	140	0.76	1.80E+05
N76D/S103A/V104I/L135F	390	0.69	5.70E+05
N76D/S103A/V104I/L135I	110	0.73	1.50E+05
N76D/S103A/V104I/L135V	140	0.86	1.60E+05
N76D/S103A/V104I/S156E*	170	2.6	6.50E+04
N76D/S103A/V104I/S166D*	160	3.5	4.60E+04
N76D/S103A/V104I/D197E	510	1.4	3.60E+05
N76D/S103A/V104I/N204A*	530	1.1	4.80E+05
N76D/S103A/V104I/N204G*	580	1.4	4.10E+05
N76D/S103A/V104I/N204C*	370	1.3	2.90E+05
N76D/S103A/V104I/P210I*	500	1.2	4.20E+05
N76D/S103A/V104I/L217H*	80	0.63	1.30E+05
N76D/S103A/V104I/M222A	70	3.1	2.30E+04
N76D/S103A/V104I/M222S	80	3.1	2.60E+04
N76D/S103A/V104I/T260P	660	1.5	4.40E+05
N76D/S103A/V104I/S265N	590	1.3	4.50E+05
K27R/N76D/V104Y/I107V/N123S	220	1.4	1.60E+05
K27R/N76D/V104Y/N123S/D197E	430	1.1	3.90E+05
K27R/N76D/V104Y/N123S/N204C	400	1.1	3.60E+05
K27R/N76D/V104Y/N123S/Q206L	440	1.2	3.70E+05



K27R/N76D/V104Y/N123S/S216V	440	1.2	3.70E+05
K27R/N76D/V104Y/N123S/N218S	760	0.98	7.80E+05
K27R/N76D/V104Y/N123S/T260P	410	1.2	3.40E+05
K27R/N76D/V104Y/N123S/T274A	390	1	3.90E+05
N76D/S103A/V104I/L126F/S265N	170	2.1	8.10E+04
N76D/S103A/V104I/S156E/S166D*	40	6.3	6.40E+03
K27R/N76D/V104Y/N123S/G195E/G197E	410	0.98	4.20E+05
K27R/N76D/V104Y/N123S/G195E/N218S	540	0.66	8.20E+05
K27R/N76D/V104Y/N123S/D197E/N218S	770	0.79	9.80E+05
K27R/N76D/V104Y/N123S/N204C/N218S	610	0.99	6.20E+05
K27R/N76D/V104Y/N123S/Q206L/N218S	580	0.78	7.40E+05
K27R/N76D/V104Y/N123S/N218S/T260P	660	1	6.60E+05
K27R/N76D/V104Y/N123S/N218S/T274A	590	0.89	6.60E+05
K27R/N76D/V104Y/Q109S/N123S/N218S/T274A	520	1	5.20E+05
K27R/N76D/V104Y/N123S/G195E/D197E/N218S	460	0.65	7.10E+05
B. amyloliquefaciens subtilisin (BPN')	50	0.14	3.60E+05
BPN'-N76D/Y217L*	380	0.46	8.30E+05

\* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

5

Table VI

Enzyme	Thermal Stability (% half-life of native enzyme)
B. lentus subtilisin	100
N76D	590
N76D/S99D	840
N76D/S103A	390
N76D/V104I	660
N76D/I107V	710
N76D/N123S	70
N76D/S99D/S101R	610
N76D/S99D/S103A	590
N76D/S99D/V104I	910
N76D/S101R/S103A	930
N76D/S101R/V104I	500
N76D/S103A/V104I	460
N76D/S103G/V104I*	370
N76D/S103A/V104F	480
N76D/S103A/V104N	230
N76D/S103A/V104S	230
N76D/S103A/V104T	370

N76D/S103A/V104W	280
N76D/S103A/V104Y	400
N76D/V104I/I107V	940
N76D/V104Y/I107V	820
N76D/V104I/N123S	80
N76D/I107V/N123S	150
K27R/N76D/V104Y/N123S	100
N76D/S99D/S101R/S103A	570
N76D/S99D/S101R/V104I	1000
N76D/S99D/S103A/V104I	680
N76D/S101G/S103A/V104I*	390
N76D/S101R/S103A/V104I	470
N76D/S103A/V104I/S105A*	360
N76D/S103A/V104I/S105D*	370
N76D/S103A/V104T/I107A*	270
N76D/S103A/V104T/I107L*	230
N76D/S103A/V104I/N123S	110
N76D/V104I/I107V/N123S	220
N76D/S103A/V104I/L126A	270
N76D/S103A/V104I/L126F	950
N76D/S103A/V104I/L126I	410
N76D/S103A/V104I/L126V	320
N76D/S103A/V104I/S128G*	640
N76D/S103A/V104I/S128L*	760
N76D/S103A/V104I/L135A	230
N76D/S103A/V104I/L135F	200
N76D/S103A/V104I/L135I	510
N76D/S103A/V104I/L135V	500
N76D/S103A/V104I/S156E*	120
N76D/S103A/V104I/S166D*	590
N76D/S103A/V104I/D197E	460
N76D/S103A/V104I/N204A*	230
N76D/S103A/V104I/N204G*	240
N76D/S103A/V104I/N204C*	500
N76D/S103A/V104I/P210I*	1370
N76D/S103A/V104I/L217H*	60
N76D/S103A/V104I/M222A	520
N76D/S103A/V104I/M222S	490
N76D/S103A/V104I/T260P	490
N76D/S103A/V104I/S265N	360
K27R/N76D/V104Y/I107V/N123S	210
K27R/N76D/V104Y/N123S/D197E	120
K27R/N76D/V104Y/N123S/N204C	110
K27R/N76D/V104Y/N123S/Q206L	380
K27R/N76D/V104Y/N123S/S216V	140
K27R/N76D/V104Y/N123S/N218S	270
K27R/N76D/V104Y/N123S/T260P	40
K27R/N76D/V104Y/N123S/T274A	60
N76D/S99D/S101R/S103A/V104I	590
N76D/S99D/S103A/V104I/N123S	110
N76D/S103A/V104I/L126F/S265N	810

N76D/S103A/V104I/S156E/S166D*	220
K27R/N76D/V104Y/N123S/G195E/G197E	90
K27R/N76D/V104Y/N123S/G195E/N218S	250
K27R/N76D/V104Y/N123S/D197E/N218S	270
K27R/N76D/V104Y/N123S/N204C/N218S	460
K27R/N76D/V104Y/N123S/Q206L/N218S	1400
K27R/N76D/V104Y/N123S/N218S/T260P	310
K27R/N76D/V104Y/N123S/N218S/T274A	180
N76D/S99D/S101R/S103A/V104I/N123S	90
K27R/N76D/V104Y/Q109S/N123S/N218S/T274	230
K27R/N76D/V104Y/N123S/G195E/D197E/N21	240
 B. amyloliquefaciens subtilisin (BPN')	 100
BPN'-N76D/Y217L*	420

\* These mutants are made as per Oligonucleotide-Directed Mutagenesis with Single-Stranded DNA Template Generated from Phagemid, all others made as per Oligonucleotide-Directed Mutagenesis, hereinbefore.

## 2. Cleaning Composition Materials:

5       The cleaning compositions of the present invention also comprise, in addition to the protease enzyme described hereinbefore, one or more cleaning composition materials compatible with the protease enzyme. The term "cleaning composition materials", as used herein, means any liquid, solid or gaseous material selected for the particular type of cleaning composition  
10       desired and the form of the product (e.g., liquid; granule; spray composition), which materials are also compatible with the protease enzyme used in the composition. The specific selection of cleaning composition materials are readily made by considering the surface, item or fabric to be cleaned, and the desired form of the composition for the cleaning conditions during use (e.g.,  
15       through the wash detergent use). The term "compatible", as used herein, means the cleaning composition materials do not reduce the proteolytic activity of the protease enzyme to such an extent that the protease is not effective as desired during normal use situations. Specific cleaning composition materials are exemplified in detail hereinafter.

20       An effective amount of one or more protease enzymes described above are included in compositions useful for cleaning a variety of surfaces in need of proteinaceous stain removal. Such cleaning compositions include detergent compositions for cleaning hard surfaces, unlimited in form (e.g., liquid and granular); detergent compositions for cleaning fabrics, unlimited in form (e.g.,  
25       granular, liquid and bar formulations); dishwashing compositions (unlimited in form); oral cleaning compositions, unlimited in form (e.g., dentifrice, toothpaste and mouthwash formulations); and denture cleaning compositions, unlimited in

form (e.g., liquid, tablet). As used herein, "effective amount of protease enzyme" refers to the quantity of protease enzyme described hereinbefore necessary to achieve the enzymatic activity necessary in the specific cleaning composition. Such effective amounts are readily ascertained by one of  
5 ordinary skill in the art and is based on many factors, such as the particular enzyme variant used, the cleaning application, the specific composition of the cleaning composition, and whether a liquid or dry (e.g., granular, bar) composition is required, and the like.

Preferably the cleaning compositions of the present invention comprise  
10 from about 0.0001% to about 10% of one or more protease enzymes, more preferably from about 0.001% to about 1%, more preferably still from about 0.001% to about 0.1%. Several examples of various cleaning compositions wherein the protease enzymes may be employed are discussed in further detail below. All parts, percentages and ratios used herein are by weight unless  
15 otherwise specified.

As used herein, "non-fabric cleaning compositions" include hard surface cleaning compositions, dishwashing compositions, oral cleaning compositions, denture cleaning compositions and personal cleansing compositions.

A. Cleaning Compositions for Hard Surfaces, Dishes and Fabrics

20 The protease enzymes can be used in any detergent composition where high sudsing and/or good insoluble substrate removal are desired. Thus the protease enzymes can be used with various conventional ingredients to provide fully-formulated hard-surface cleaners, dishwashing compositions, fabric laundering compositions and the like. Such compositions can be in the  
25 form of liquids, granules, bars and the like. Such compositions can be formulated as modern "concentrated" detergents which contain as much as 30%-60% by weight of surfactants.

The cleaning compositions herein can optionally, and preferably, contain various anionic, nonionic, zwitterionic, etc., surfactants. Such surfactants are  
30 typically present at levels of from about 0.1% to about 60%, preferably from about 1% to about 35%, of the compositions.

Nonlimiting examples of surfactants useful herein include the conventional C<sub>11</sub>-C<sub>18</sub> alkyl benzene sulfonates and primary and random alkyl sulfates, the C<sub>10</sub>-C<sub>18</sub> secondary (2,3) alkyl sulfates of the formulas  
35  $\text{CH}_3(\text{CH}_2)_x(\text{CHOSO}_3^- \text{M}^+)\text{CH}_3$  and  $\text{CH}_3(\text{CH}_2)_y(\text{CHOSO}_3^- \text{M}^+)\text{CH}_2\text{CH}_3$  wherein x and (y+1) are integers of at least about 7, preferably at least about 9, and M is a water-solubilizing cation, especially sodium, the C<sub>10</sub>-C<sub>18</sub> alkyl

alkoxy sulfates (especially EO 1-7 ethoxy sulfates), C<sub>10</sub>-C<sub>18</sub> alkyl alkoxy carboxylates (especially the EO 1-7 ethoxycarboxylates), the C<sub>10</sub>-C<sub>18</sub> alkyl polyglycosides, and their corresponding sulfated polyglycosides, C<sub>12</sub>-C<sub>18</sub> alpha-sulfonated fatty acid esters, C<sub>12</sub>-C<sub>18</sub> alkyl and alkyl phenol alkoxyates (especially ethoxylates and mixed ethoxy/propoxy), C<sub>12</sub>-C<sub>18</sub> betaines and sulfobetaines ("sultaines"), C<sub>10</sub>-C<sub>18</sub> amine oxides, C<sub>8</sub>-C<sub>24</sub> sarcosinates (especially oleoyl sarcosinate), and the like. The alkyl alkoxy sulfates (AES) and alkyl alkoxy carboxylates (AEC) are preferred herein. (Use of such surfactants in combination with the aforesaid amine oxide and/or betaine or sultaine surfactants is also preferred, depending on the desires of the formulator.) Other conventional useful surfactants are listed in standard texts. Particularly useful surfactants include the C<sub>10</sub>-C<sub>18</sub> N-methyl glucamides disclosed in US Patent 5, 194,639, Connor et al., issued March 16, 1993, incorporated herein by reference.

Particularly useful is the class of nonionic surfactants which are condensates of ethylene oxide with a hydrophobic moiety to provide a surfactant having an average hydrophilic-lipophilic balance (HLB) in the range from 5 to 17, preferably from 6 to 14, more preferably from 7 to 12. The hydrophobic (lipophilic) moiety may be aliphatic or aromatic in nature and the length of the polyoxyethylene group which is condensed with any particular hydrophobic group can be readily adjusted to yield a water-soluble compound having the desired degree of balance between hydrophilic and hydrophobic elements. Especially preferred are the C<sub>9</sub>-C<sub>15</sub> primary alcohol ethoxylates (or mixed ethoxy/propoxy) containing 3-8 moles of ethylene oxide per mole of alcohol, particularly the C<sub>14</sub>-C<sub>15</sub> primary alcohols containing 6-8 moles of ethylene oxide per mole of alcohol, the C<sub>12</sub>-C<sub>15</sub> primary alcohols containing 3-5 moles of ethylene oxide per mole of alcohol, and mixtures thereof.

A wide variety of other ingredients useful in detergent cleaning compositions can be included in the compositions herein, including other active ingredients, carriers, hydrotropes, processing aids, dyes or pigments, solvents for liquid formulations, etc. If an additional increment of sudsing is desired, suds boosters such as the C<sub>10</sub>-C<sub>16</sub> alkylamidates can be incorporated into the compositions, typically at about 1% to about 10% levels. The C<sub>10</sub>-C<sub>14</sub> monoethanol and diethanol amides illustrate a typical class of such suds boosters. Use of such suds boosters with high sudsing adjunct surfactants such as the amine oxides, betaines and sultaines noted above is also advantageous. If desired, soluble magnesium salts such as MgCl<sub>2</sub>, MgSO<sub>4</sub>,

and the like, can be added at levels of, typically, from about 0.1% to about 2%, to provide additional sudsing.

The liquid detergent compositions herein can contain water and other solvents as carriers. Low molecular weight primary or secondary alcohols exemplified by methanol, ethanol, propanol, and isopropanol are suitable. Monohydric alcohols are preferred for solubilizing surfactants, but polyols such as those containing from about 2 to about 6 carbon atoms and from about 2 to about 6 hydroxy groups (e.g., 1,3-propanediol, ethylene glycol, glycerine, and 1,2-propanediol) can also be used. The compositions may contain from about 5% to about 90%, typically from about 10% to about 50% of such carriers.

The detergent compositions herein will preferably be formulated such that during use in aqueous cleaning operations, the wash water will have a pH between about 6.8 and about 11.0. Finished products thus are typically formulated at this range. Techniques for controlling pH at recommended usage levels include the use of buffers, alkalis, acids, etc., and are well known to those skilled in the art.

When formulating the hard surface cleaning compositions and fabric cleaning compositions of the present invention, the formulator may wish to employ various builders at levels from about 5% to about 50% by weight. Typical builders include the 1-10 micron zeolites, polycarboxylates such as citrate and oxydisuccinates, layered silicates, phosphates, and the like. Other conventional builders are listed in standard formularies.

Likewise, the formulator may wish to employ various additional enzymes, such as cellulases, lipases, amylases, peroxidases, and proteases in such compositions, typically at levels of from about 0.001% to about 1% by weight. Various deterative and fabric care enzymes are well-known in the laundry detergent art.

Various bleaching compounds, such as the percarbonates, perborates and the like, can be used in such compositions, typically at levels from about 1% to about 15% by weight. If desired, such compositions can also contain bleach activators such as tetraacetyl ethylenediamine, nonanoyloxybenzene sulfonate, and the like, which are also known in the art. Usage levels typically range from about 1% to about 10% by weight.

Various soil release agents, especially of the anionic oligoester type, various chelating agents, especially the aminophosphonates and ethylenediaminedisuccinates, various clay soil removal agents, especially ethoxylated tetraethylene pentamine, various dispersing agents, especially

polyacrylates and polyasparatates, various brighteners, especially anionic brighteners, various dye transfer inhibiting agents, such as polyvinyl pyrrolidone, various suds suppressors, especially silicones and secondary alcohols, various fabric softeners, especially smectite clays and clay flocculating polymers (e.g., poly(oxy ethylene)), and the like can all be used in such compositions at levels ranging from about 1% to about 35% by weight. Standard formularies and published patents contain multiple, detailed descriptions of such conventional materials.

Enzyme stabilizers may also be used in the cleaning compositions of the present invention. Such enzyme stabilizers include propylene glycol (preferably from about 1% to about 10%), sodium formate (preferably from about 0.1% to about 1%) and calcium formate (preferably from about 0.1% to about 1%).

1. Hard surface cleaning compositions

As used herein "hard surface cleaning composition" refers to liquid and granular detergent compositions for cleaning hard surfaces such as floors, walls, bathroom tile, and the like. Hard surface cleaning compositions of the present invention comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 5%, more preferably still from about 0.001% to about 1% by weight of active protease enzyme of the composition. In addition to comprising one or more protease enzymes, such hard surface cleaning compositions typically comprise a surfactant and a water-soluble sequestering builder. In certain specialized products such as spray window cleaners, however, the surfactants are sometimes not used since they may produce a filmy/streaky residue on the glass surface.

The surfactant component, when present, may comprise as little as 0.1% of the compositions herein, but typically the compositions will contain from about 0.25% to about 10%, more preferably from about 1% to about 5% of surfactant.

Typically the compositions will contain from about 0.5% to about 50% of a detergency builder, preferably from about 1% to about 10%. Preferably the pH should be in the range of about 8 to 12. Conventional pH adjustment agents such as sodium hydroxide, sodium carbonate or hydrochloric acid can be used if adjustment is necessary.

Solvents may be included in the compositions. Useful solvents include, but are not limited to, glycol ethers such as diethyleneglycol monohexyl ether,

diethyleneglycol monobutyl ether, ethyleneglycol monobutyl ether, ethyleneglycol monohexyl ether, propyleneglycol monobutyl ether, dipropyleneglycol monobutyl ether, and diols such as 2,2,4-trimethyl-1,3-pentanediol and 2-ethyl-1,3-hexanediol. When used, such solvents are typically present at levels of from about 0.5% to about 15%, preferably from about 3% to about 11%.

Additionally, highly volatile solvents such as isopropanol or ethanol can be used in the present compositions to facilitate faster evaporation of the composition from surfaces when the surface is not rinsed after "full strength" application of the composition to the surface. When used, volatile solvents are typically present at levels of from about 2% to about 12% in the compositions.

The hard surface cleaning composition embodiment of the present invention is illustrated by the following nonlimiting examples. (In the following examples, reference to "Protease #" in the examples is to the variant useful in the present invention compositions having the given number in Table III hereinbefore.)

Examples 1-6  
Liquid Hard Surface Cleaning Compositions

		Example No.					
20	Component	1	2	3	4	5	6
	Protease # 12	0.05	0.20	0.02	0.03	0.10	0.03
	Protease # 4	—	—	—	—	0.20	0.02
	EDTA**	—	—	2.90	2.90	—	—
	Na Citrate	—	—	—	—	2.90	2.90
25	NaC <sub>12</sub> Alkyl-benzene sulfonate	1.95	—	1.95	—	1.95	—
	NaC <sub>12</sub> Alkylsulfate	—	2.20	—	2.20	—	2.20
	NaC <sub>12</sub> (ethoxy)*** sulfate	—	2.20	—	2.20	—	2.20
30	C <sub>12</sub> Dimethylamine oxide	—	0.50	—	0.50	—	0.50
	Na Cumene sulfonate	1.30	—	1.30	—	1.30	—
	Hexyl Carbitol***	6.30	6.30	6.30	6.30	6.30	6.30
	Water****	balance to 100%					

35 \*\*Na<sub>4</sub> ethylenediamine diacetic acid

\*\*\*Diethyleneglycol monohexyl ether

\*\*\*\*All formulas adjusted to pH 7



In Examples 1-4 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

- 5 In Examples 5 and 6, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

#### Examples 7-12

#### 10 Spray Compositions for Cleaning Hard Surfaces and Removing Household Mildew

		Example No.					
Component		7	8	9	10	11	12
15	Protease # 12	0.20	0.05	0.10	0.30	0.20	0.30
	Protease # 4	-	-	-	-	0.30	0.10
	Sodium octyl sulfate	2.00	2.00	2.00	2.00	2.00	2.00
	Sodium dodecyl sulfate	4.00	4.00	4.00	4.00	4.00	4.00
	Sodium hydroxide	0.80	0.80	0.80	0.80	0.80	0.80
20	Silicate (Na)	0.04	0.04	0.04	0.04	0.04	0.04
	Perfume	0.35	0.35	0.35	0.35	0.35	0.35
	Water	balance to 100%					

Product pH is about 7.

- 25 In Examples 7-10 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

- 30 In Examples 11 and 12, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

#### 2. Dishwashing Compositions

- 35 In another embodiment of the present invention, dishwashing compositions comprise one or more protease enzymes. As used herein, "dishwashing composition" refers to all forms for compositions for cleaning dishes, including but not limited to, granular and liquid forms. The dishwashing composition embodiment of the present invention is illustrated by the following examples.

Examples 13-18  
Dishwashing Composition

		Example No.					
Component		13	14	15	16	17	18
5	Protease # 12	0.05	0.50	0.02	0.40	0.10	0.03
	Protease # 4	-	-	-	-	0.40	0.02
	C <sub>12</sub> -C <sub>14</sub> N-methyl-glucamide	0.90	0.90	0.90	0.90	0.90	0.90
	C <sub>12</sub> ethoxy (1) sulfate	12.00	12.00	12.00	12.00	12.00	12.00
10	2-methyl undecanoic acid	4.50	4.50	-	4.50	4.50	-
	C <sub>12</sub> ethoxy (2) carboxylate	4.50	4.50	4.50	4.50	4.50	4.50
	C <sub>12</sub> alcohol ethoxylate (4)	3.00	3.00	3.00	3.00	3.00	3.00
	C <sub>12</sub> amine oxide	3.00	3.00	3.00	3.00	3.00	3.00
	Sodium cumene sulfonate	2.00	2.00	2.00	2.00	2.00	2.00
15	Ethanol	4.00	4.00	4.00	4.00	4.00	4.00
	Mg <sup>++</sup> (as MgCl <sub>2</sub> )	0.20	0.20	0.20	0.20	0.20	0.20
	Ca <sup>++</sup> (as CaCl <sub>2</sub> )	0.40	0.40	0.40	0.40	0.40	0.40
	Water	balance to 100%					

Product pH is adjusted to 7.

- 20 In Examples 13-16 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

- 25 In Examples 17 and 18, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4 with substantially similar results.

Example 19Granular Automatic Dishwashing Composition

<u>C</u> mponent	<u>A</u>	<u>B</u>	<u>C</u>
Citric Acid	15.0	-	-
Citrate	4.0	29.0	15.0
Acrylate/methacrylate copolymer	6.0	-	6.0
Acrylic acid maleic acid copolymer	-	3.7	-
Dry add carbonate	9.0	-	20.0
Alkali metal silicate	8.5	17.0	9.0
Paraffin	-	0.5	-
Benzotriazole	-	0.3	-
Termamyl 60T	1.5	1.5	1.0
Protease # 12 (4.6% prill)	1.6	1.6	1.6
Percarbonate (AvO)	1.5	-	-
Perborate monohydrate	-	0.3	1.5
Perborate tetrahydrate	-	0.9	-
Tetraacetylene diamine	3.8	4.4	-
Diethylene triamine penta methyl phosphonic acid (Mg salt)	0.13	0.13	0.13
Alkyl ethoxy sulphate - 3 times ethoxylated	3.0	-	-
Alkyl ethoxy propoxy nonionic surfactant	-	1.5	-
Suds suppressor	2.0	-	-
Olin SLF18 nonionic surfactant	-	-	2.0
Sulphate	Balance to 100%		

- 5 In Examples 19 A-C the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease #12, with substantially similar results. Also in Examples 19 A-C, any combination of the proteases useful in the present invention recited in Tables III, V and VI among  
10 others, are substituted for Protease # 12 with substantially similar results.

3. Fabric cleaning compositions

- In another embodiment of the present invention, fabric cleaning compositions comprise one or more protease enzymes. As used herein, "fabric cleaning composition" refers to all forms for detergent compositions for  
15 cleaning fabrics, including but not limited to, granular, liquid and bar forms.

a. Granular fabric cleaning compositions

The granular fabric cleaning compositions of the present invention contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.005% to about 5%, more preferably from about 0.01% to about 1% by weight of active protease enzyme of the composition. In addition to one or more protease enzymes, the granular fabric cleaning compositions typically comprise at least one surfactant, one or more builders, and, in some cases, a bleaching agent.

The granular fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 20-23

Granular Fabric Cleaning Composition

		Example No.			
<u>Component</u>		<u>20</u>	<u>21</u>	<u>22</u>	<u>23</u>
15	Protease # 12 (4% Prill)	0.10	0.20	0.03	0.05
	Protease # 4 (4% Prill)	-	-	0.02	0.05
	C <sub>13</sub> linear alkyl benzene sulfonate	22.00	22.00	22.00	22.00
	Phosphate (as sodium tripolyphosphates)	23.00	23.00	23.00	23.00
20	Sodium carbonate	23.00	23.00	23.00	23.00
	Sodium silicate	14.00	14.00	14.00	14.00
	Zeolite	8.20	8.20	8.20	8.20
	Chelant (diethylenetriamine-pentaacetic acid)	0.40	0.40	0.40	0.40
25	Sodium sulfate	5.50	5.50	5.50	5.50
	Water	balance to 100%			

In Examples 20-21 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 22 and 23, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 24-27  
Granular Fabric Cleaning Composition

		Example No.			
	Component	24	25	26	27
5	Protease # 12 (4% Prill)	0.10	0.20	0.03	0.05
5	Protease # 4 (4% Prill)	-	-	0.02	0.05
	C <sub>12</sub> alkyl benzene sulfonate	12.00	12.00	12.00	12.00
	Zeolite A (1-10 micrometer)	26.00	26.00	26.00	26.00
	2-butyl octanoic acid	4.00	4.00	4.00	4.00
10	C <sub>12</sub> -C <sub>14</sub> secondary (2,3) alkyl sulfate, Na salt	5.00	5.00	5.00	5.00
	Sodium citrate	5.00	5.00	5.00	5.00
	Optical brightener	0.10	0.10	0.10	0.10
	Sodium sulfate	17.00	17.00	17.00	17.00
15	Fillers, water, minors	balance to 100%			

In Examples 24 and 25 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

- 20 In Examples 26 and 27, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 28 and 29Granular Fabric Cleaning Compositions

<u>Components</u>	<u>Example No.</u>	
	<u>28</u>	<u>29</u>
Linear alkyl benzene sulphonate	11.4	10.70
Tallow alkyl sulphate	1.80	2.40
C <sub>14-15</sub> alkyl sulphate	3.00	3.10
C <sub>14-15</sub> alcohol 7 times ethoxylated	4.00	4.00
Tallow alcohol 11 times ethoxylated	1.80	1.80
Dispersant	0.07	0.1
Silicone fluid	0.80	0.80
Trisodium citrate	14.00	15.00
Citric acid	3.00	2.50
Zeolite	32.50	32.10
Maleic acid acrylic acid copolymer	5.00	5.00
Diethylene triamine penta methylene phosphonic acid	1.00	0.20
Protease # 12 (4% Prill)	0.30	0.30
Lipase	0.36	0.40
Amylase	0.30	0.30
Sodium silicate	2.00	2.50
Sodium sulphate	3.50	5.20
Polyvinyl pyrrolidone	0.30	0.50
Perborate	0.5	1
Phenol sulphonate	0.1	0.2
Peroxidase	0.1	0.1
Minors	Up to 100	Up to 100

5

Examples 30 and 31Granular Fabric Cleaning Compositions

<u>Components</u>	<u>Example No.</u>	
	<u>30</u>	<u>31</u>
Sodium linear C <sub>12</sub> alkyl benzene-sulfonate	6.5	8.0
Sodium sulfate	15.0	18.0
Zeolite A	26.0	22.0
Sodium nitrilotriacetate	5.0	5.0
Polyvinyl pyrrolidone	0.5	0.7
Tetraacetylene diamine	3.0	3.0

Boric acid	4.0	-
Perborate	0.5	1
Phenol sulphonat	0.1	0.2
Protease # 12 (4% Prill)	0.4	0.4
Fillers (e.g., silicates; carbonates; perfumes; water)	Up to 100	Up to 100

Example 32Compact Granular Fabric Cleaning Composition

<u>Components</u>	<u>Weight %</u>
Alkyl Sulphate	8.0
Alkyl Ethoxy Sulphate	2.0
Mixture of C25 and C45 alcohol 3 and 7 times ethoxylated	6.0
Polyhydroxy fatty acid amide	2.5
Zeolite	17.0
Layered silicate/citrate	16.0
Carbonate	7.0
Maleic acid acrylic acid copolymer	5.0
Soil release polymer	0.4
Carboxymethyl cellulose	0.4
Poly (4-vinylpyridine) -N-oxide	0.1
Copolymer of vinylimidazole and vinylpyrrolidone	0.1
PEG2000	0.2
Protease # 12 (4% Prill)	0.5
Lipase	0.2
Cellulase	0.2
Tetracetylene diamine	6.0
Percarbonate	22.0
Ethylene diamine disuccinic acid	0.3
Suds suppressor	3.5
Disodium-4,4'-bis (2-morpholino -4-anilino-s-triazin-6-ylamino) stilbene-2,2'-disulphonate	0.25
Disodium-4,4'-bis (2-sulfostryl) biphenyl	0.05
Water, Perfume and Minors	Up to 100

Example 33Granular Fabric Cleaning Composition

5

<u>Component</u>	<u>Weight %</u>
Linear alkyl benzene sulphonate	7.6

C <sub>16</sub> -C <sub>18</sub> alkyl sulfate	1.3
C <sub>14</sub> - <sub>15</sub> alcohol 7 times ethoxylated	4.0
Coco-alkyl-dimethyl hydroxyethyl ammonium chloride	1.4
Dispersant	0.07
Silicone fluid	0.8
Trisodium citrate	5.0
Zeolite 4A	15.0
Maleic acid acrylic acid copolymer	4.0
Diethylene triamine penta methylene phosphonic acid	0.4
Perborate	15.0
Tetraacetylene diamine	5.0
Smectite clay	10.0
Poly (oxy ethylene) (MW 300,000)	0.3
Protease # 12 (4% Prill)	0.4
Lipase	0.2
Amylase	0.3
Cellulase	0.2
Sodium silicate	3.0
Sodium carbonate	10.0
Carboxymethyl cellulose	0.2
Brighteners	0.2
Water, perfume and minors	Up to 100

Example 34Granular Fabric Cleaning Composition

<u>Component</u>	<u>Weight %</u>
Linear alkyl benzene sulfonate	6.92
Tallow alkyl sulfate	2.05
C <sub>14</sub> - <sub>15</sub> alcohol 7 times ethoxylated	4.4
C <sub>12</sub> - <sub>15</sub> alkyl ethoxy sulfate - 3 times ethoxylated	0.16
Zeolite	20.2
Citrate	5.5
Carbonate	15.4
Silicate	3.0
Maleic acid acrylic acid copolymer	4.0
Carboxymethyl cellulose	0.31
Soil release polymer	0.30
Protease # 12 (4% Prill)	0.2



Lipase	0.36
Cellulase	0.13
Perborate tetrahydrate	11.64
Perborate monohydrate	8.7
Tetraacetylene diamine	5.0
Diethylene tramine penta methyl phosphonic acid	0.38
Magnesium sulfate	0.40
Brightener	0.19
Perfume, silicone, suds suppressors	0.85
Minors	Up to 100

In each of Examples 28-34 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 28-34, any combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

b. Liquid fabric cleaning compositions

Liquid fabric cleaning compositions of the present invention comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 10%, more preferably from about 0.001% to about 1%, and most preferably from about 0.001% to about 0.1%, by weight of active protease enzyme of the composition. Such liquid fabric cleaning compositions typically additionally comprise an anionic surfactant, a fatty acid, a water-soluble detergent builder and water.

The liquid fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 35-39  
Liquid Fabric Cleaning Compositions

		Example No.				
Component		35	36	37	38	39
5	Protease # 12	0.05	0.03	0.30	0.03	0.10
	Protease # 4	—	—	—	0.01	0.20
	C <sub>12</sub> - C <sub>14</sub> alkyl sulfate, Na	20.00	20.00	20.00	20.00	20.00
	2-Butyl octanoic acid	5.00	5.00	5.00	5.00	5.00
	Sodium citrate	1.00	1.00	1.00	1.00	1.00
10	C <sub>10</sub> alcohol ethoxylate (3)	13.00	13.00	13.00	13.00	13.00
	Monethanolamine	2.50	2.50	2.50	2.50	2.50
<u>Water/propylene glycol/ethanol (100:1:1)</u>		<u>balance to 100%</u>				

In Examples 35-37 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

In Examples 38 and 39, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

Examples 40-41  
Liquid Fabric Cleaning Compositions

		<u>Example No.</u>	
<u>Component</u>		<u>40</u>	<u>41</u>
5	C <sub>12-14</sub> alkenyl succinic acid	3.0	8.0
	Citric acid monohydrate	10.0	15.0
	Sodium C <sub>12-15</sub> alkyl sulphate	8.0	8.0
	Sodium sulfate of C <sub>12-15</sub> alcohol 2 times ethoxylated	-	3.0
	C <sub>12-15</sub> alcohol 7 times ethoxylated	-	8.0
10	C <sub>12-15</sub> alcohol 5 times ethoxylated	8.0	-
	Diethylene triamine penta (methylene phosphonic acid)	0.2	-
	Oleic acid	1.8	-
	Ethanol	4.0	4.0
	Propanediol	2.0	2.0
15	Protease # 12	0.2	0.2
	Polyvinyl pyrrolidone	1.0	2.0
	Suds suppressor	0.15	0.15
	NaOH	up to pH 7.5	
	Perborate	0.5	1
20	Phenol sulphonate	0.1	0.2
	Peroxidase	0.4	0.1
	Waters and minors	up to 100 parts	

In each of Examples 40 and 41 herein the Protease #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 40 and 41, any combination of the proteases useful in the present invention recited in Tables III, V and VI, among others, are substituted from Protease # 12 with substantially similar results.

30 c. Bar fabric cleaning compositions

Bar fabric cleaning compositions of the present invention suitable for hand-washing soiled fabrics contain an effective amount of one or more protease enzymes, preferably from about 0.001% to about 10%, more preferably from about 0.01% to about 1% by weight of the composition.

35 The bar fabric cleaning composition embodiment of the present invention is illustrated by the following examples.

Exempl s 42-45  
Bar Fabric Cleaning Compositions

		Example No.			
Component		42	43	44	45
5	Protease # 12	0.3	-	0.1	0.02
	Protease # 4	-	-	0.4	0.03
	C <sub>12</sub> -C <sub>16</sub> alkyl sulfate, Na	20.0	20.0	20.0	20.00
	C <sub>12</sub> -C <sub>14</sub> N-methyl glucamide	5.0	5.0	5.0	5.00
	C <sub>11</sub> -C <sub>13</sub> alkyl benzene sulfonate, Na	10.0	10.0	10.0	10.00
10	Sodium carbonate	25.0	25.0	25.0	25.00
	Sodium pyrophosphate	7.0	7.0	7.0	7.00
	Sodium tripolyphosphate	7.0	7.0	7.0	7.00
	Zeolite A (0.1-.10 $\mu$ )	5.0	5.0	5.0	5.00
	Carboxymethylcellulose	0.2	0.2	0.2	0.20
15	Polyacrylate (MW 1400)	0.2	0.2	0.2	0.20
	Coconut monethanolamide	5.0	5.0	5.0	5.00
	Brightener, perfume	0.2	0.2	0.2	0.20
	CaSO <sub>4</sub>	1.0	1.0	1.0	1.00
	MgSO <sub>4</sub>	1.0	1.0	1.0	1.00
20	Water	4.0	4.0	4.0	4.00
	Filler*	balance to 100%			

\*Can be selected from convenient materials such as CaCO<sub>3</sub>, talc, clay, silicates, and the like.

25 In Examples 42 and 43 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results.

30 In Examples 44 and 45, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 and Protease # 4, with substantially similar results.

**B. Additional Cleaning Compositions**

35 In addition to the hard surface cleaning, dishwashing and fabric cleaning compositions discussed above, one or more protease enzymes may be incorporated into a variety of other cleaning compositions where hydrolysis of an insoluble substrate is desired. Such additional cleaning compositions

include but are not limited to, oral cleaning compositions, denture cleaning compositions, and contact lens cleaning compositions.

1. Oral cleaning compositions

- 5 In another embodiment of the present invention, a pharmaceutically-acceptable amount of one or more protease enzymes are included in compositions useful for removing proteinaceous stains from teeth or dentures. As used herein, "oral cleaning compositions" refers to dentifrices, toothpastes, toothgels, toothpowders, mouthwashes, mouth sprays, mouth gels, chewing gums, lozenges, sachets, tablets, biogels, prophylaxis pastes, dental treatment solutions, and the like. Preferably, oral cleaning compositions of the present invention comprise from about 0.0001% to about 20% of one or more protease enzymes, more preferably from about 0.001% to about 10%, more preferably still from about 0.01% to about 5%, by weight of the composition, and a pharmaceutically-acceptable carrier. As used herein, "pharmaceutically-acceptable" means that drugs, medicaments or inert ingredients which the term describes are suitable for use in contact with the tissues of humans and lower animals without undue toxicity, incompatibility, instability, irritation, allergic response, and the like, commensurate with a reasonable benefit/risk ratio.

- 15 Typically, the pharmaceutically-acceptable oral cleaning carrier components of the oral cleaning components of the oral cleaning compositions will generally comprise from about 50% to about 99.99%, preferably from about 65% to about 99.99%, more preferably from about 65% to about 99%, by weight of the composition.

- 20 The pharmaceutically-acceptable carrier components and optional components which may be included in the oral cleaning compositions of the present invention are well known to those skilled in the art. A wide variety of composition types, carrier components and optional components useful in the oral cleaning compositions are disclosed in U.S. Patent 5,096,700, Seibel, issued March 17, 1992; U.S. Patent 5,028,414, Sampathkumar, issued July 2, 1991; and U.S. Patent 5,028,415, Benedict, Bush and Sunberg, issued July 2, 1991; all of which are incorporated herein by reference.

30 The oral cleaning composition embodiment of the present invention is illustrated by the following examples.

Examples 46-49  
Dentifrice Composition

	Component	Example No.			
		46	47	48	49
5	Protease # 12	2.000	3.500	1.500	2.000
	Sorbitol (70% aqueous solution)	35.000	35.000	35.000	35.000
	PEG-6*	1.000	1.000	1.000	1.000
	Silica dental abrasive**	20.000	20.000	20.000	20.000
	Sodium fluoride	0.243	0.243	0.243	0.243
10	Titanium dioxide	0.500	0.500	0.500	0.500
	Sodium saccharin	0.286	0.286	0.286	0.286
	Sodium alkyl sulfate (27.9% aqueous solution)	4.000	4.000	4.000	4.000
	Flavor	1.040	1.040	1.040	1.040
15	Carboxyvinyl Polymer***	0.300	0.300	0.300	0.300
	Carrageenan****	0.800	0.800	0.800	0.800
	Water	balance to 100%			

\*PEG-6 = Polyethylene glycol having a molecular weight of 600.

\*\*Precipitated silica identified as Zeodent 119 offered by J.M. Huber.

\*\*\*Carbopol offered by B.F. Goodrich Chemical Company.

\*\*\*\*Iota Carrageenan offered by Hercules Chemical Company.

In Examples 46-49 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 46-49, any combination of the protease enzymes useful in the present invention recited in Tables III, V, VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 50-53  
Mouthwash Composition

		Example No.			
	Component	50	51	52	53
5	Protease # 12	3.00	7.50	1.00	5.00
	SDA 40 Alcohol	8.00	8.00	8.00	8.00
	Flavor	0.08	0.08	0.08	0.08
	Emulsifier	0.08	0.08	0.08	0.08
	Sodium Fluoride	0.05	0.05	0.05	0.05
10	Glycerin	10.00	10.00	10.00	10.00
	Sweetener	0.02	0.02	0.02	0.02
	Benzoic acid	0.05	0.05	0.05	0.05
	Sodium hydroxide	0.20	0.20	0.20	0.20
	Dye	0.04	0.04	0.04	0.04
15	Water	balance to 100%			

In Examples 50-53 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 50-53, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

Examples 54-57  
Lozenge Composition

		Example No.			
	Component	54	55	56	57
25	Protease # 12	0.01	0.03	0.10	0.02
	Sorbitol	17.50	17.50	17.50	17.50
	Mannitol	17.50	17.50	17.50	17.50
30	Starch	13.60	13.60	13.60	13.60
	Sweetener	1.20	1.20	1.20	1.20
	Flavor	11.70	11.70	11.70	11.70
	Color	0.10	0.10	0.10	0.10
	Corn Syrup	balance to 100%			

In Examples 54-57 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with

substantially similar results. Also in Examples 54-57, any combination of the protease enzymes useful in the present invention recited in Tables III, V and VI, among others, are substituted for Protease # 12 with substantially similar results.

5

Examples 58-61  
Chewing Gum Composition

		Example No.			
Component		58	59	60	61
	Protease # 12	0.03	0.02	0.10	0.05
10	Sorbitol crystals	38.44	38.40	38.40	38.40
	Paloja-T gum base*	20.00	20.00	20.00	20.00
	Sorbitol (70% aqueous solution)	22.00	22.00	22.00	22.00
	Mannitol	10.00	10.00	10.00	10.00
	Glycerine	7.56	7.56	7.56	7.56
15	Flavor	1.00	1.00	1.00	1.00

\*Supplied by L.A. Dreyfus Company.

In Examples 58-61 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 58-61, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

2. Denture cleaning compositions

In another embodiment of the present invention, denture cleaning compositions for cleaning dentures outside of the oral cavity comprise one or more protease enzymes. Such denture cleaning compositions comprise an effective amount of one or more protease enzymes, preferably from about 0.0001% to about 50% of one or more protease enzymes, more preferably from about 0.001% to about 35%, more preferably still from about 0.01% to about 20%, by weight of the composition, and a denture cleansing carrier. Various denture cleansing composition formats such as effervescent tablets and the like are well known in the art (see for example U.S. Patent 5,055,305, Young, incorporated herein by reference), and are generally appropriate for incorporation of one or more protease enzymes for removing proteinaceous stains from dentures.



The denture cleaning composition embodiment of the present invention is illustrated by the following examples.

#### Examples 62-65

##### Two-layer Effervescent Denture Cleansing Tablet

5	Component	Example No.			
		62	63	64	65
	<u>Acidic Layer</u>				
	Protease # 12	1.0	1.5	0.01	0.05
	Tartaric acid	24.0	24.0	24.00	24.00
10	Sodium carbonate	4.0	4.0	4.00	4.00
	Sulphamic acid	10.0	10.0	10.00	10.00
	PEG 20,000	4.0	4.0	4.00	4.00
	Sodium bicarbonate	24.5	24.5	24.50	24.50
	Potassium persulfate	15.0	15.0	15.00	15.00
15	Sodium acid pyrophosphate	7.0	7.0	7.00	7.00
	Pyrogenic silica	2.0	2.0	2.00	2.00
	Tetracetylene diamine	7.0	7.0	7.00	7.00
	Ricinoleylsulfosuccinate	0.5	0.5	0.50	0.50
	Flavor	1.0	1.0	1.00	1.00
20	<u>Alkaline Layer</u>				
	Sodium perborate monohydrate	32.0	32.0	32.00	32.00
	Sodium bicarbonate	19.0	19.0	19.00	19.00
	EDTA	3.0	3.0	3.00	3.00
	Sodium tripolyphosphate	12.0	12.0	12.00	12.00
25	PEG 20,000	2.0	2.0	2.00	2.00
	Potassium persulfate	26.0	26.0	26.00	26.00
	Sodium carbonate	2.0	2.0	2.00	2.00
	Pyrogenic silica	2.0	2.0	2.00	2.00
	Dye/flavor	2.0	2.0	2.00	2.00

30 In Examples 62-65 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 62-65, any combination of the protease enzymes useful in the present invention recited in Tables III, V and

35 VI, among others, are substituted for Protease # 12 with substantially similar results.

### 3. Personal Cleansing Compositions

In another embodiment of the present invention, personal cleaning compositions for cleaning the skin (in liquid and bar form) comprise one or more of the protease enzymes. Such compositions typically comprise from about 0.001% to about 5% protease enzyme, preferably from about 0.005% to about 2%, and most preferably from about 0.01% to about 0.8% by weight of the composition. Preferred personal cleansing compositions into which can be included protease enzymes as described herein are taught in U.S. Patent Applications SN 08/121,623 and SN 08/121,624, both by Visscher et al., filed September 14, 1993, the disclosures of which are incorporated herein by reference in their entirety. Such compositions are illustrated by the following examples.

Example 66Liquid Personal Cleansing Compositions Containing Soap

	<u>Component</u>	<u>Weight %</u>
15	Soap (K or Na)	15.00
	30% Laurate	
	30% Myristate	
	25% Palmitate	
	15% Stearate	
20	Fatty acids (above ratios)	4.50
	Na Lauryl Sarcosinate	6.00
	Sodium Laureth-3 Sulfate	0.66
	Cocamidopropylbetaine	1.33
	Glycerine	15.00
25	Propylene glycol	9.00
	Polyquaternium 10	0.80
	Ethylene glycol distearate (EDTA)	1.50
	Propylparaben	0.10
	Methylparaben	0.20
30	Protease # 12	0.10
	KOH or NaOH	If necessary to adjust pH
	Calcium sulfate	3
	Acetic acid	3
	Water	Balance to 100

Example 67Personal Cleansing Bar Composition

<u>Component</u>	<u>Weight %</u>
------------------	-----------------

	Sodium Cocoyl Isethionate	47.20
	Sodium C tearyl Sulfate	9.14
	Paraffin	9.05
	Sodium Soap (in situ)	3.67
5	Sodium Isethionate	5.51
	Sodium Chloride	0.45
	Titanium Dioxide	0.4
	Trisodium EDTA	0.1
	Trisodium Etidronate	0.1
10	Perfume	1.20
	Na <sub>2</sub> SO <sub>4</sub>	0.87
	Protease # 12	0.10
	Water/Minors	Balance to 100

In Examples 66-67 the Proteases #'s 1-11 and 13-25 recited in Table III, among others including the additional proteases useful in the present invention described in Tables V and VI, are substituted for Protease # 12, with substantially similar results. Also in Examples 66-67, any combination of the protease enzymes useful in the present invention recited in Tables III, V, and VI, among others, are substituted for Protease # 12 with substantially similar results.

#### Example 68

##### Wash Performance Test

The wash performance of the variants useful in the present invention compositions is evaluated by measuring the removal of stain from EMPA 116 (blood/milk/carbon black on cotton) cloth swatches (Testfabrics, Inc., Middlesex, NJ 07030).

Six EMPA 116 swatches, cut to 3 X 4-1/2 inches with pinked edges, are placed in each pot of a Model 7243S Terg-O-Tometer (United States Testing Co., Inc., Hoboken, NJ) containing 1000 ml of water, 15 gpg hardness (Ca<sup>++</sup>:Mg<sup>++</sup>::3:1::w:w), 7 g of detergent, and enzyme as appropriate. The detergent base is WFK1 detergent from wfk - Testgewebe GmbH, Adlerstrasse 42, Postfach 13 07 62, D-47759 Krefeld, Germany:

Component	% of Final Formulation
Zeolite A	25%
Sodium sulfate	25%
Soda Ash	10%
Linear alkylbenzenesulfonate	8.8%

Alcohol thoxylate (7-8 EO)	4.5%
Sodium soap	3%
Sodium silicate (SiO <sub>2</sub> :Na <sub>2</sub> O::3.3:1)	3%

To this base detergent, the following additions are made:

Component	% of Final Formulation
Sodium perborate monohydrate	13%
Copolymer (Sokalan CP5)	4%
TAED (Mykon ATC Green)	3%
Enzyme	0.5%
Brightener (Tinopal AMS-GX)	0.2%

- 5 Sodium perborate monohydrate is obtained from Degussa Corporation, Ridgefield-Park, NJ 07660. Sokalan CP5 is obtained from BASF Corporation, Parsippany, NJ 07054. Mykon ATC Green (TAED, tetraacetylenediamine) is obtained from Warwick International, Limited, Mostyn, Holywell, Clwyd CH8 9HE, England. Tinopal AMS GX is obtained from Ciba-Geigy Corporation, 10 Greensboro, NC 27419.

- 15 Six EMPA 116 swatches are washed in detergent with enzyme for 30 min at 60°C and are subsequently rinsed twice for 5 minutes each time in 1000 ml water. Enzymes are added at final concentrations of 0.05 to 1 ppm for standard curves, and 0.25 ppm for routine analyses. Swatches are dried and 15 pressed, and the reflectance from the swatches is measured using the L value on the L\*a\*b\* scale of a Minolta Chroma Meter, Model CR-200 (Minolta Corporation, Ramsey, NJ 07446). Performance is reported as a percentage of the performance of *B. lentus* (GG36) protease and is calculated by dividing the amount of *B. lentus* (GG36) protease by the amount of variant protease that is 20 needed to provide the same stain removal performance X 100. The data are shown in Table VII.

Table VII

Enzyme	Wash Performance
<i>B. lentus</i> subtilisin	100
N76D	310
N76D/S103A	230
N76D/V104I	130
N76D/V107V	160
N76D/S99D/S101R	370
N76D/S99D/S103A	290
N76D/S101R/S103A	130
N76D/S101R/V104I	300
N76D/S103A/V104I	320
N76D/S103G/V104I	160
N76D/S103A/V104F	210
N76D/S103A/V104N	110

N76D/S103A/V104T	170
N76D/V104I/I107V	210
N76D/S99D/S101R/S103A	220
N76D/S99D/S101R/V104I	140
N76D/S101G/S103A/V104I	170
N76D/S101R/S103A/V104I	150
N76D/S103A/V104I/S105A	170
N76D/S103A/V104T/I107A	120
N76D/S103A/V104T/I107L	110
N76D/S103A/V104I/L126F	110
N76D/S103A/V104I/S128G	280
N76D/S103A/V104I/L135I	160
N76D/S103A/V104I/L135V	160
N76D/S103A/V104I/D197E	170
N76D/S103A/V104I/N204A	160
N76D/S103A/V104I/N204G	150
N76D/S103A/V104I/P210I	470
N76D/S103A/V104I/M222A	100
N76D/S103A/V104I/T260P	280
N76D/S103A/V104I/S265N	190

#### Example 69

##### Protease Stability in a Liquid Detergent Formulation

- A comparison of protease stability toward inactivation in a liquid detergent formulation is made for *Bacillus lentus* subtilisin and its variant enzyme N76D/S103A/V104I according to the procedure outlined herein. The detergent formulation for the study is a commercially purchased bottle of Tide Ultra liquid laundry detergent made in the USA by The Procter & Gamble Company. Heat treatment of the detergent formulation is necessary to inactivate *in-situ* protease. This is accomplished by incubating the detergent at 96°C for a period of 4.5 hours. Concentrated preparations of the *B. lentus* subtilisin and N76D/S103A/V104I variant, in the range of 20 grams/liter enzyme, are then added to the heat-treated Tide Ultra at room-temperature to a final concentration of 0.3 grams/liter enzyme in the detergent formulation. The heat-treated detergent with protease added is then incubated in a water bath thermostatted at 50°C. Aliquots are removed from the incubation tubes at 0, 24, 46, 76, and 112 hour time intervals and assayed for enzyme activity by addition to a 1 cm cuvette containing 1.2 mM of the synthetic peptide substrate suc-Ala-Ala-Pro-phe-p-nitroanilide dissolved in 0.1M tris-HCL buffer, pH 8.6, and thermostatted at 25°C. The initial linear reaction velocity is followed spectrophotometrically by monitoring the absorbance of the reaction product p-nitroaniline at 410nm as a function of time. As shown in Fig. 10, the N76D/S103A/V104I variant is observed to have significantly greater stability towards inactivation than the native *B. lentus* enzyme. Estimated half-lives for inactivation in the Tide Ultra detergent formulation for the two enzymes, under

the specified test conditions, are 45 hours for *B. lentus* subtilisin and 125 hours for the N76D/S103A/V104I variant.

5 While particular embodiments of the subject invention have been described, it will be obvious to those skilled in the art that various changes and modifications of the subject invention can be made without departing from the spirit and scope of the invention. It is intended to cover, in the appended claims, all such modifications that are within the scope of the invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Baeck, Andre (NMN)  
Ghosh, Chanchal K.  
Graycar, Thomas P.  
Bott, Richard R.  
Wilson, Lori J.  
Brode, Philip F., III  
Barnett, Bobby L.  
Rubingh, Donn N.

(ii) TITLE OF INVENTION: Protease-Containing Cleaning Compositions

(iii) NUMBER OF SEQUENCES: 15

## (iv) CORRESPONDENCE ADDRESS:

- (A) ADDRESSEE: The Procter & Gamble Company  
(B) STREET: 11810 East River Road  
(C) CITY: Cincinnati  
(D) STATE: OH  
(E) COUNTRY: USA  
(F) ZIP: 45253-8707

## (v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:  
(B) FILING DATE: 13-OCT-1994  
(C) CLASSIFICATION:

## (viii) ATTORNEY/AGENT INFORMATION:

- (A) NAME: Zerby, Kim William  
(B) REGISTRATION NUMBER: 32,323  
(C) REFERENCE/DOCKET NUMBER: 5040R

## (ix) TELECOMMUNICATION INFORMATION:

- (A) TELEPHONE: (513) 627-2885  
(B) TELEFAX: (513) 627-0318

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GAAGCTGCAA CTCGTAAA

19

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GCTGCTCTAG ACAATTCG

18

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 39 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTATTAGGGG CGGACGGTCG AGGCGCCATC AGCTCGATT

39

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TCAGGTTTCGG TCTCGAGCGT TGCCCAAGGA TTG

33

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CACGTTGCTA GCTTGAGTTT AG

22

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1497 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GGTCTACTAA AATATTATTC CATACTATAC AATTAATACA CAGAATAATC TGTCTATTGG

60

TTATTCTGCA AATGAAAAAA AGGAGAGGAT AAAGAGTGAG AGGCAAAAAA GTATGGATCA

120



```

GTTTGCTGTT TGCTTTAGCG TTAATCTTTA CGATGGCGTT CGGCAGCACA TCCTCTGCCC 180
AGGCGGCAGG GAAATCAAAC GGGGAAAAGA AATATATTGT CGGGTTTAAA CAGACAATGA 240
GCACGATGAG CGCCGCTAAG AAGAAAGATG TCATTCTGA AAAAGGCGGG AAAGTGCAAA 300
AGCAATTCRA ATATGTAGAC GCAGCTTCAG TCACATTAAA CGAAAAGCT GTAAAAGAAT 360
TGAAAAAGA CCCGAGCGTC GCTTACGTTG AAGAAGATCA CGTAGCACAT GCGTACGCGC 420
AGTCCGTGCC TTACGGCGTA TCACAAATTA AAGCCCCCTGC TCTGCACTCT CAAGGCTACA 480
CTGGATCAAA TGTTAAAGTA GCGGTTATCG ACAGCGGTAT CGATTCTTCT CATCCTGATT 540
TAAAGGTAGC AAGCGGAGCC AGCATGGTTC CTTCTGA AAC AAATCCTTTC CAAGACAACA 600
ACTCTCACGG AACTCACGTT GCCGGCACAG TTGCGGCTCT TAATAACTCA ATCGGTGTAT 660
TAGGCGTTGC GCCAAGCGCA TCACTTTACG CTGTAAGAAGT TCTCGGTGCT GACGGTTCCG 720
GCCAATACAG CTGGATCATT AACGGAATCG AGTGGGCGAT CGCAAACAAT ATGGACGTTA 780
TTAACATGAG CCTCGGCGGA CCTTCTGGTT CTGCTGCTTT AAAAGCGGCA GTTGATAAAG 840
CCGTTGCATC CGGCGTCGTA GTCGTTGCGG CAGCCGCTAA CGAAGGCACT TCCGGCAGCT 900
CAAGCACAGT GGGTACCCTT GGTAAATACC CTTCTGTCAT TGCAGTAGGC GCTGTTGACA 960
GCAGCAACCA AAGAGCATCT TTCTAAGCG TAGGACCTGA GCTTGATGTC ATGGCACCTG 1020
GCGTATCTAT CCAAAGCAGC CTTCTGGAA ACAATACGG GCGGTACAAC GGTACGTCAA 1080
TGGCATCTCC GCACGTTGCC GGAGCGGCTG CTTTGATTCT TTCTAAGCAC CCGAACTGGA 1140
CAAACACTCA AGTCCGCAGC AGTTTAGAAA ACACCACTAC AAAACTTGGT GATTCTTTGT 1200
ACTATGGAAA AGGGCTGATC AACGTACAAG CGGCAGCTCA GTAAACATA AAAAACCGGC 1260
CTTGCCCCCG CCGGTTTTTT ATTATTTTTC TTCCTCCGCA TGTTCAATCC GCTCCATAAT 1320
CGACGGATGG CTCCCTCTGA AAATTTTAAC GAGAAACGGC GGGTTGACCC GGCTCAGTCC 1380
CGTAACGGCC AACTCCTGAA ACGTCTCAAT CGCCGCTTCC CGGTTTCCGG TCAGCTCAAT 1440
GCCATAACGG TCGGCGGCGT TTTCTGATA CCGGGAGACG GCATTGCTAA TCGGATC 1497

```

## (2) INFORMATION FOR SEQ ID NO:7:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Ala Gln Ser Val Pro Tyr Gly Val Ser Gln Ile Lys Ala Pro Ala Leu
1           5           10           15
His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp
20        25        30
Ser Gly Ile Asp Ser Ser His Pro Asp Leu Lys Val Ala Gly Gly Ala
35        40        45

```

60

Ser Met Val Pro Ser Glu Thr Asn Pro Phe Gln Asp Asn Asn Ser His  
 50 55 60  
 Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn Ser Ile Gly  
 65 70 75 80  
 Val Leu Gly Val Ala Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu  
 85 90 95  
 Gly Ala Asp Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu  
 100 105 110  
 Trp Ala Ile Ala Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly  
 115 120 125  
 Pro Ser Gly Ser Ala Ala Leu Lys Ala Ala Val Asp Lys Ala Val Ala  
 130 135 140  
 Ser Gly Val Val Val Val Ala Ala Ala Gly Asn Glu Gly Thr Ser Gly  
 145 150 155 160  
 Ser Ser Ser Thr Val Gly Tyr Pro Gly Lys Tyr Pro Ser Val Ile Ala  
 165 170 175  
 Val Gly Ala Val Asp Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Val  
 180 185 190  
 Gly Pro Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr  
 195 200 205  
 Leu Pro Gly Asn Lys Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Ser  
 210 215 220  
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn  
 225 230 235 240  
 Trp Thr Asn Thr Gln Val Arg Ser Ser Leu Glu Asn Thr Thr Thr Lys  
 245 250 255  
 Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala  
 260 265 270  
 Ala Ala Gln  
 275

## (2) INFORMATION FOR SEQ ID NO:8:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 275 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Ala Gln Ser Val Pro Tyr Gly Ile Ser Gln Ile Lys Ala Pro Ala Leu  
 1 5 10 15  
 His Ser Gln Gly Tyr Thr Gly Ser Asn Val Lys Val Ala Val Ile Asp  
 20 25 30  
 Ser Gly Ile Asp Ser Ser His Pro Asp Leu Asn Val Arg Gly Gly Ala  
 35 40 45  
 Ser Phe Val Pro Ser Glu Thr Asn Pro Tyr Gln Asp Gly Ser Ser His  
 50 55 60  
 Gly Thr His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly  
 65 70 75 80

Val Leu Gly Val Ser Pro Ser Ala Ser Leu Tyr Ala Val Lys Val Leu  
 85 90 95  
 Asp Ser Thr Gly Ser Gly Gln Tyr Ser Trp Ile Ile Asn Gly Ile Glu  
 100 105 110  
 Trp Ala Ile Ser Asn Asn Met Asp Val Ile Asn Met Ser Leu Gly Gly  
 115 120 125  
 Pro Thr Gly Ser Thr Ala Leu Lys Thr Val Val Asp Lys Ala Val Ser  
 130 135 140  
 Ser Gly Ile Val Val Ala Ala Ala Ala Gly Asn Glu Gly Ser Ser Gly  
 145 150 155 160  
 Ser Thr Ser Thr Val Gly Tyr Pro Ala Lys Tyr Pro Ser Thr Ile Ala  
 165 170 175  
 Val Gly Ala Val Asn Ser Ser Asn Gln Arg Ala Ser Phe Ser Ser Ala  
 180 185 190  
 Gly Ser Glu Leu Asp Val Met Ala Pro Gly Val Ser Ile Gln Ser Thr  
 195 200 205  
 Leu Pro Gly Gly Thr Tyr Gly Ala Tyr Asn Gly Thr Ser Met Ala Thr  
 210 215 220  
 Pro His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Thr  
 225 230 235 240  
 Trp Thr Asn Ala Gln Val Arg Asp Arg Leu Glu Ser Thr Ala Thr Tyr  
 245 250 255  
 Leu Gly Asn Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Gln Ala  
 260 265 270  
 Ala Ala Gln  
 275

## (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 274 amino acids
  - (B) TYPE: amino acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Ala Gln Thr Val Pro Tyr Gly Ile Pro Leu Ile Lys Ala Asp Lys Val  
 1 5 10 15  
 Gln Ala Gln Gly Phe Lys Gly Ala Asn Val Lys Val Ala Val Leu Asp  
 20 25 30  
 Thr Gly Ile Gln Ala Ser His Pro Asp Leu Asn Val Val Gly Gly Ala  
 35 40 45  
 Ser Phe Val Ala Gly Glu Ala Tyr Asn Thr Asp Gly Asn Gly His Gly  
 50 55 60  
 Thr His Val Ala Gly Thr Val Ala Ala Leu Asp Asn Thr Thr Gly Val  
 65 70 75 80  
 Leu Gly Val Ala Pro Ser Val Ser Leu Tyr Ala Val Lys Val Leu Asn  
 85 90 95

62

Ser Ser Gly Ser Gly Ser Tyr Ser Gly Ile Val Ser Gly Ile Glu Trp  
 100 105 110  
 Ala Thr Thr Asn Gly Met Asp Val Ile Asn Met Ser Leu Gly Gly Ala  
 115 120 125  
 Ser Gly Ser Thr Ala Met Lys Gln Ala Val Asp Asn Ala Tyr Ala Arg  
 130 135 140  
 Gly Val Val Val Val Ala Ala Ala Gly Asn Ser Gly Asn Ser Gly Ser  
 145 150 155 160  
 Thr Asn Thr Ile Gly Tyr Pro Ala Lys Tyr Asp Ser Val Ile Ala Val  
 165 170 175  
 Gly Ala Val Asp Ser Asn Ser Asn Arg Ala Ser Phe Ser Ser Val Gly  
 180 185 190  
 Ala Glu Leu Glu Val Met Ala Pro Gly Ala Gly Val Tyr Ser Thr Tyr  
 195 200 205  
 Pro Thr Asn Thr Tyr Ala Thr Leu Asn Gly Thr Ser Met Ala Ser Pro  
 210 215 220  
 His Val Ala Gly Ala Ala Ala Leu Ile Leu Ser Lys His Pro Asn Leu  
 225 230 235 240  
 Ser Ala Ser Gln Val Arg Asn Arg Leu Ser Ser Thr Ala Thr Tyr Leu  
 245 250 255  
 Gly Ser Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn Val Glu Ala Ala  
 260 265 270  
 Ala Gln

## (2) INFORMATION FOR SEQ ID NO:10:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 269 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Gln Ser Val Pro Trp Gly Ile Ser Arg Val Gln Ala Pro Ala Ala  
 1 5 10 15  
 His Asn Arg Gly Leu Thr Gly Ser Gly Val Lys Val Ala Val Leu Asp  
 20 25 30  
 Thr Gly Ile Ser Thr His Pro Asp Leu Asn Ile Arg Gly Gly Ala Ser  
 35 40 45  
 Phe Val Pro Gly Glu Pro Ser Thr Gln Asp Gly Asn Gly His Gly Thr  
 50 55 60  
 His Val Ala Gly Thr Ile Ala Ala Leu Asn Asn Ser Ile Gly Val Leu  
 65 70 75 80  
 Gly Val Ala Pro Ser Ala Glu Leu Tyr Ala Val Lys Val Leu Gly Ala  
 85 90 95  
 Ser Gly Ser Gly Ser Val Ser Ser Ile Ala Gln Gly Leu Glu Trp Ala  
 100 105 110

63

Gly Asn Asn Gly Met His Val Ala Asn Leu Ser Leu Gly Ser Pro Ser  
 115 120 125

Pro Ser Ala Thr Leu Glu Gln Ala Val Asn Ser Ala Thr Ser Arg Gly  
 130 135 140

Val Leu Val Val Ala Ala Ser Gly Asn Ser Gly Ala Gly Ser Ile Ser  
 145 150 155 160

Tyr Pro Ala Arg Tyr Ala Asn Ala Met Ala Val Gly Ala Thr Asp Gln  
 165 170 175

Asn Asn Asn Arg Ala Ser Phe Ser Gln Tyr Gly Ala Gly Leu Asp Ile  
 180 185 190

Val Ala Pro Gly Val Asn Val Gln Ser Thr Tyr Pro Gly Ser Thr Tyr  
 195 200 205

Ala Ser Leu Asn Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ala  
 210 215 220

Ala Ala Leu Val Lys Gln Lys Asn Pro Ser Trp Ser Asn Val Gln Ile  
 225 230 235 240

Arg Asn His Leu Lys Asn Thr Ala Thr Ser Leu Gly Ser Thr Asn Leu  
 245 250 255

Tyr Gly Ser Gly Leu Val Asn Ala Glu Ala Ala Thr Arg  
 260 265

## (2) INFORMATION FOR SEQ ID NO:11:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCGCAC TACTCATTTC TGTTGCTTTT 60

AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTTAAT TGGCTTTAAT 120

GAGCAGGAAG CTGTCACTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTGCGCCATT 180

CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCCCTGTT 240

TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTCTT 300

TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGGAAATTAGC 360

CGTGTGCAAG CCCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420

GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480

GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540

ATTGCTGCTT TAAACAATTC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAACATATC 600

GCTGTAAAG TATTAGGGGC GAGCGGTTCA GGITCGGTCA GCTCGATTGC CCAAGGATTG 660

GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTGA GTTTAGGAAG CCCTTCGCCA 720

AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTTGTAGCG 780

GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840

64

GCAGTCGGAG CTA CTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900  
 CTTGACATTG TCGCACCAGG TGTAACCGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960  
 AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020  
 CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080  
 AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1140 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: DNA (genomic)

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATGAAGAAAC CGTTGGGGAA AATTGTCGCA AGCACCAGC TACTCATTTC TGTGCTTTT 60  
 AGTTCATCGA TCGCATCGGC TGCTGAAGAA GCAAAAGAAA AATATTAAAT TGGCTTTAAT 120  
 GAGCAGGAAG CTGTCAGTGA GTTTGTAGAA CAAGTAGAGG CAAATGACGA GGTCGCCATT 180  
 CTCTCTGAGG AAGAGGAAGT CGAAATTGAA TTGCTTCATG AATTTGAAAC GATTCTCTGT 240  
 TTATCCGTTG AGTTAAGCCC AGAAGATGTG GACGCGCTTG AACTCGATCC AGCGATTCTT 300  
 TATATTGAAG AGGATGCAGA AGTAACGACA ATGGCGCAAT CAGTGCCATG GGAATTAGC 360  
 CGTGTGCAAG CCCAGCTGC CCATAACCGT GGATTGACAG GTTCTGGTGT AAAAGTTGCT 420  
 GTCCTCGATA CAGGTATTTT CACTCATCCA GACTTAAATA TTCGTGGTGG CGCTAGCTTT 480  
 GTACCAGGGG AACCATCCAC TCAAGATGGG AATGGGCATG GCACGCATGT GGCCGGGACG 540  
 ATTGCTGCTT TAGACAATC GATTGGCGTT CTTGGCGTAG CGCCGAGCGC GGAATATAC 600  
 GCTGTAAAG TATTAGGGGC GAGCGGTTCA GCGCCATCA GCTCGATTGC CCAAGGATTG 660  
 GAATGGGCAG GGAACAATGG CATGCACGTT GCTAATTTGA GTTTAGGAAG CCCTTCGCCA 720  
 AGTGCCACAC TTGAGCAAGC TGTTAATAGC GCGACTTCTA GAGGCGTTCT TGTGTAGCG 780  
 GCATCTGGGA ATTCAGGTGC AGGCTCAATC AGCTATCCGG CCCGTTATGC GAACGCAATG 840  
 GCAGTCGGAG CTA CTGACCA AAACAACAAC CGCGCCAGCT TTTCACAGTA TGGCGCAGGG 900  
 CTTGACATTG TCGCACCAGG TGTAACCGTG CAGAGCACAT ACCCAGGTTC AACGTATGCC 960  
 AGCTTAAACG GTACATCGAT GGCTACTCCT CATGTTGCAG GTGCAGCAGC CCTTGTTAAA 1020  
 CAAAAGAACC CATCTTGGTC CAATGTACAA ATCCGCAATC ATCTAAAGAA TACGGCAACG 1080  
 AGCTTAGGAA GCACGAACTT GTATGGAAGC GGACTTGTCA ATGCAGAAGC GGCAACACGC 1140

## (2) INFORMATION FOR SEQ ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TATGCCAGCC ACAACGGTAC TTCGATGGCT

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 31 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CACAGTTGCG GCTCTAGATA ACTCAATCGG T

31

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 33 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GCTGACGGTT CCGGCGCTAT TAGTTGGATC ATT

33

What is claimed is:

1. A cleaning composition comprising:
  - (a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin; and
  - (b) one or more cleaning composition materials compatible with the protease enzyme.
2. The cleaning compositions according to Claim 1 wherein the cleaning composition materials are selected from the group consisting of surfactants, solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, builders, bleaching agents, dyes, perfumes, and mixtures thereof.
3. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least about 1% surfactant by weight of the composition, said surfactant comprising materials selected from the group consisting of alkyl benzene sulfonates, primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxylates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof.
4. The cleaning composition according to Claim 3 further comprising at least about 5% builder selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.



5. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one bleaching agent.

6. The cleaning compositions according to Claim 5 wherein the bleaching agent is selected from the group consisting of percarbonates, perborates, and mixtures thereof, and optionally further comprising at least one bleach activator.

7. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases and mixtures thereof.

8. The cleaning compositions according to Claim 1 wherein the cleaning composition materials comprise at least one fabric softener.

9. The cleaning compositions according to Claim 1 wherein the precursor carbonyl hydrolase for the protease enzyme is a subtilisin, and the protease enzyme is a subtilisin variant wherein a combination of substitutions is made at the positions equivalent to 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/126, 76/103/104/135, 76/103/104/197, 76/103/104/222, 76/103/104/260, 76/103/104/265, 76/103/104/126/265, 27/76/104/123/274, 27/76/104/109/123/274, 27/76/104/123/218/274, 27/76/104/123, 27/76/104/107/123, 27/76/104/109/123, 27/76/104/109/123/218/274, 27/76/104/123/197, 27/76/104/123/204, 27/76/104/123/206, 27/76/104/123/216, 27/76/104/123/218, 27/76/104/123/260, 27/76/104/123/195/197, 27/76/104/123/195/218, 27/76/104/123/197/218, 27/76/104/123/204/218, 27/76/104/123/206/218, 27/76/104/123/218/260, 27/76/104/123/195/197/218, 76/103/104/217, 76/103/104/156, 76/103/104/166, 76/103/104/105, 76/101/103/104, 76/103/104/128, 76/103/104/210, 76/103/104/107, 76/103/104/204, 76/217, 76/103/104/156/166 and 76/103/104/128.

10. The cleaning composition according to Claim 9 wherein the protease enzyme is a subtilisin variant selected from the group consisting of 76/99, 76/101, 76/103, 76/104, 76/107, 76/123, 76/99/101, 76/99/103, 76/99/104, 76/101/103, 76/101/104, 76/103/104, 76/104/107, 76/104/123, 76/107/123, 5 76/99/101/103, 76/99/101/104, 76/99/103/104, 76/101/103/104, 76/103/104/123, 76/104/107/123, 76/99/101/103/104, 76/99/103/104/123, 76/99/101/103/104/123, 76/103/104/128; 76/103/104/260; 76/103/104/265; 76/103/104/197; 76/103/104/105; 76/103/104/135; 76/103/104/126; 76/103/104/107; 76/103/104/210; 76/103/104/126/265; and/or 76/103/104/222.

11. A fabric cleaning composition comprising:  
 (a) an effective amount of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; 5 N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; 10 N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/S128G; 15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N and N76D/S103A/V104I/M222A and mixtures thereof; and  
 20 (b) one or more cleaning composition materials compatible with the protease enzyme comprising at least about 5% surfactant and at least about 5% builder, by weight of the composition.

12. The fabric cleaning composition according to Claim 11 further comprising cleaning composition materials selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds

boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

13. The fabric cleaning composition according to Claim 11 further comprising at least one bleaching agent.

14. The fabric cleaning composition according to Claim 11 further comprising at least one enzyme selected from the group consisting of cellulases, lipases, amylases, proteases, peroxidases, and mixtures thereof.

15. The fabric cleaning composition according to Claim 11 in the form of a liquid, granule or bar.

16. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a subtilisin variant selected from N76D/S99D, N76D/V104I, N76D/S99D/V104I, N76D/S103A/V104I, N76D/V104I/I107V, N76D/V104Y/I107V, N76D/S101R/S103A/V104I, N76D/S99D/S101R/S103A/V104I, N76D/S101R/V104I, and mixtures thereof.

17. The fabric cleaning composition according to Claim 11 wherein the protease enzyme is a Bacillus subtilisin variant selected from N76D/V104I, N76D/S103A/V104I, and mixtures thereof.

18. A fabric cleaning composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from Bacillus lentus subtilisin;

(b) at least about 5% surfactant;

(c) at least about 5% builder; and

(d) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, soil release agents, clay soil removal agents, dispersing agents, brighteners, suds suppressors, fabric softeners, suds boosters, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

19. The fabric cleaning composition according to Claim 18 wherein the surfactant is selected from the group consisting of alkyl benzene sulfonates,

primary alkyl sulfates, secondary alkyl sulfates, alkyl alkoxy sulfates, alkyl alkoxy carboxylates, alkyl polyglycosides and their corresponding sulfated polyglycosides, alpha-sulfonated fatty acid esters, alkyl and alkyl phenol alkoxyates, betaines and sulfobetaines, amine oxides, N-methyl glucamides, nonionic primary alcohol ethoxylates, nonionic primary alcohol mixed ethoxy/propoxy, and mixtures thereof; and wherein further the builder is selected from the group consisting of zeolites, polycarboxylates, layered silicates, phosphates, and mixtures thereof.

20. The fabric cleaning composition according to Claim 19 further comprising one or more cleaning composition materials selected from the group consisting of bleaching agents, fabric softeners, and enzymes.

21. The fabric cleaning composition according to Claim 18 in the form of a concentrated granular fabric cleaning composition comprising at least about 30% surfactant.

22. A dishwashing composition comprising:

(a) from about 0.0001% to about 10% protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and N76D/S103A/V104I/M222A and mixtures thereof;

(b) from about 0.1% to about 10% surfactant; and

(c) optionally, one or more cleaning composition materials compatible with the protease enzyme selected from the group consisting of solvents, buffers, enzymes, dispersing agents, suds suppressors, enzyme stabilizers, bleaching agents, dyes, perfumes, and mixtures thereof.

23. The dishwashing composition according to Claim 22 wherein the protease enzyme is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

24. A personal cleansing composition comprising:

(a) from about 0.001% to about 5% by weight of protease enzyme which is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a precursor carbonyl hydrolase comprising a substitution of a different amino acid for a plurality of amino acid residues at a position in said precursor carbonyl hydrolase equivalent to position +76 in *Bacillus amyloliquefaciens* subtilisin, in combination with one or more amino acid residue positions equivalent to those selected from the group consisting of +99, +101, +103, +104, +107, +123, +27, +105, +109, +126, +128, +135, +156, +166, +195, +197, +204, +206, +210, +216, +217, +218, +222, +260, +265, and/or +274 in *Bacillus amyloliquefaciens* subtilisin;

(b) from about 0.01% to about 95% by weight of a surfactant system; and

(c) optionally, from about 0.05% to about 50% of an enzyme stabilizer.

25. The composition of Claim 24 wherein the protease enzyme is at a level of from about 0.001% to about 2% by weight.

26. The composition of Claim 25 wherein the protease enzyme is at a level of from about 0.01% to about 0.8% by weight.

27. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of anionic carboxylates, amine oxides, alkyl glucosides, glucose amides, alkyl sulfates, alkyl ether sulfates, acyl isethionates, alkyl sulfosuccinates, alkyl phosphate esters, ethoxylated phosphate esters, alkyl glyceryl ether sulfonates, or mixtures thereof.

28. The composition of Claim 24 wherein the surfactant system comprises a surfactant selected from the group consisting of soaps, acylglutamates, alkyl

sarcosinates, lauramine oxides, cocamine oxides, cocamidopropylamine oxides, decylglucosides, lauryl sulfates, laureth sulfates, C<sub>12</sub>-C<sub>18</sub> acyl isethionates, or mixtures thereof.

29. The composition of Claim 28 wherein the surfactant is soap at a level of at least about 2% by weight of the composition.

30. The composition of Claim 29 wherein the soap is at a level of at least about 10% by weight of the composition.

31. The composition of Claim 30 wherein the soap is at a level of at least about 25% by weight of the composition.

32. The composition of Claim 28 wherein the ratio of soap to protease enzyme is from about 2,000:1 to about 8:1.

33. The composition of Claim 32 wherein the ratio of soap to protease enzyme is from about 400:1 to about 40:1.

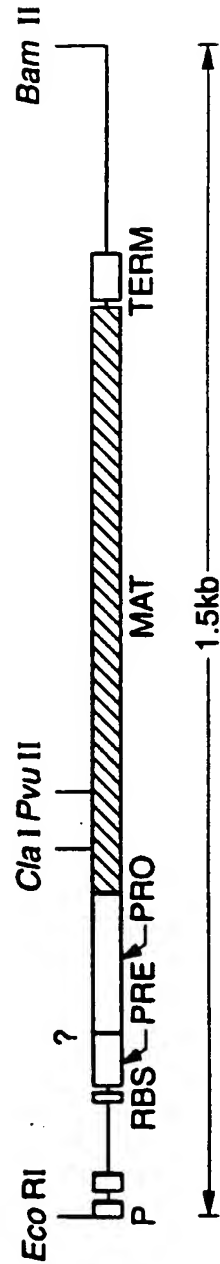
34. The composition of Claim 28 wherein the protease enzyme is a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D;  
5 N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S;  
N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I;  
N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I;  
N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S;  
N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I;  
10 N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I;  
N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S;  
N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S;  
N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G;  
15 N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N;  
N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A;  
N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F;  
N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N, and  
N76D/S103A/V104I/M222A; and mixtures thereof.

35. A method for cleaning fabric, said method comprising contacting a fabric in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

36. A method for cleaning dishes, said method comprising contacting a dish in need of cleaning with a protease enzyme which is an N76D/S103A/V104I subtilisin variant derived from *Bacillus lentus* subtilisin.

37. A method for personal cleansing, said method comprising contacting the part of the human or lower animal body in need of cleaning with a carbonyl hydrolase variant having an amino acid sequence not found in nature, which is derived from a subtilisin precursor carbonyl hydrolase, and wherein the protease enzyme is a subtilisin variant selected from N76D/S99D; N76D/S101R; N76D/S103A; N76D/V104I; N76D/I107V; N76D/N123S; N76D/S99D/S101R; N76D/S99D/S103A; N76D/S99D/V104I; N76D/S101R/S103A; N76D/S101R/V104I; N76D/S103A/V104I; N76D/V104I/I107V; N76D/V104Y/I107V; N76D/V104I/N123S; N76D/I107V/N123S; N76D/S99D/S101R/S103A; N76D/S99D/S101R/V104I; N76D/S99D/S103A/V104I; N76D/S101R/S103A/V104I; N76D/S103A/V104I/N123S; N76D/V104I/I107V/N123S; N76D/S99D/S101R/S103A/V104I; N76D/S99D/S103A/V104I/N123S; N76D/S99D/S101R/S103A/V104I/N123S; N76D/S103A/V104I/S128G; N76D/S103A/V104I/T260P; N76D/S103A/V104I/S265N; N76D/S103A/V104I/D197E; N76D/S103A/V104I/S105A; N76D/S103A/V104I/L135I; N76D/S103A/V104I/L126F; N76D/S103A/V104T/L107T; N76D/S103A/V104I/L126F/S265N and N76D/S103A/V104I/M222A; and mixtures thereof.

1 / 20

**FIG. 1A**



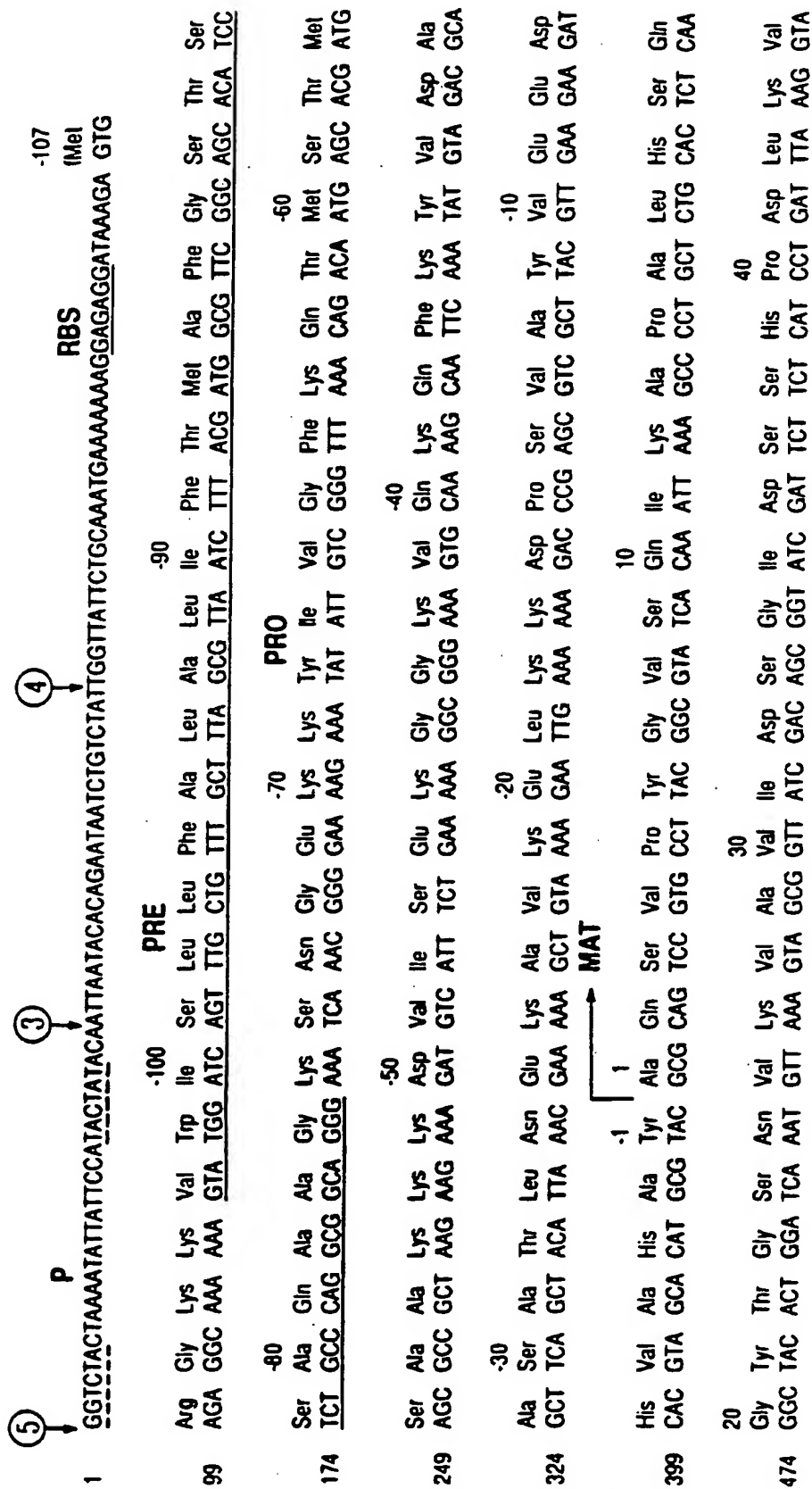


FIG..1B - 1



4 / 20

250 Gln Gln Asn Thr Thr Thr Lys Leu Gly Asp Ser Phe Tyr Tyr Gly Lys Gly Leu Ile Asn  
 1149 CAA GTC CGC AGC AGT TTA GAA AAC AAC ACC ACT ACA AAA CTT GGT GAT ICT TTC TTC TAC TAT GGA AAA GGG CIG AIC AAC  
 270 Val Gln Ala Ala Ala Gln OC  
 1224 GTA CAG GCG GCA GCT CAG TAA AACATAAAAAACCGGCGCTTGGCCCCCGCGGTTTTTATTTTTCTTCCTCGCAIGTTCAATCGGCTCC  
 1316 ATAATCGACGGATGGCTCCCTCTGAAAAATTTTAACGAGAAACGGCGGGTTGACCCGCTCAGTCCCGTAACGGCCAAGTCCTGAAACGCTCAATCGGCCG  
 1416 CTCCCGGTTCCGGTCAGCTCAATGCCGTAACGGTCGGCGGTTTCCTGATACGGGAGACGGCATTCGTAAATCGGATC

**FIG.\_1B - 3****FIG.\_1B**

FIG.\_1B - 1

FIG.\_1B - 2

FIG.\_1B - 3

5/20

CONSERVED RESIDUES IN SUBTILISINS FROM  
*BACILLUS AMYLOLIQUEFACIENS*

1	10	20
A Q S V P . G . . . . .	A P A . H . .	G
21	30	40
. T G S . V K V A V . D . G . . . .		H P
41	50	60
D L . . . G G A S . V P . . . . .		Q D
61	70	80
. N . H G T H V A G T . A A L N N S I G		
81	90	100
V L G V A P S A . L Y A V K V L G A . G		
101	110	120
S G . . S . L . . G . E W A . N . . . .		
121	130	140
V . N . S L G . P S . S . . . . .		A . .
141	150	160
. . . . . G V . V V A A . G N . G . . .		
161	170	180
. . . . . Y P . . Y . . . . .		A V G A .
181	190	200
D . . N . . A S F S . . G . . L D . . A		
201	210	220
P G V . . Q S T . P G . . Y . . . .		N G T
221	230	240
S M A . P H V A G A A A L . . . .		K . . .
241	250	260
W . . . Q . R . . L . N T . . . .		L G . .
261	270	
. . Y G . G L . N . . A A . . .		

**FIG.\_2**

SUBSTITUTE SHEET (RULE 26)

6/20

## COMPARISON OF SUBTILISIN SEQUENCES FROM:

*B. amyloliquefaciens**B. subtilis**B. licheniformis**B. lentus*

01	A	Q	S	V	P	Y	G	V	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P				
	A	Q	S	V	P	Y	G	I	S	Q	I	K	A	P	A	L	H	S	Q	G	Y	T	G	S	N	V	K	V	A	V	I	D	S	G	I	D	S	S	H	P				
	A	Q	T	V	P	Y	G	I	P	L	I	K	A	D	K	V	Q	A	Q	G	F	K	G	A	N	V	K	V	A	V	L	D	T	G	I	Q	A	S	H	P				
	A	Q	S	V	P	W	G	I	S	R	V	Q	A	P	A	A	H	N	R	G	L	T	G	S	G	V	K	V	A	V	L	D	T	G	I	S	T	*	H	P				
41	D	D	L	K	V	A	G	G	A	S	M	V	P	S	E	T	N	P	P	Q	D	N	N	S	H	G	T	H	V	A	G	T	V	A	A	L	N	N	S	I	G			
	D	D	L	N	V	R	G	G	A	S	F	V	P	S	E	T	N	P	Y	Q	D	G	S	S	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G			
	D	D	L	N	V	V	G	G	A	S	F	V	A	G	E	A	Y	N	*	T	D	G	N	G	H	G	T	H	V	A	G	T	V	A	A	L	D	N	T	T	G			
	D	D	L	N	I	R	G	G	A	S	F	V	P	G	E	*	P	S	T	Q	D	G	N	G	H	G	T	H	V	A	G	T	I	A	A	L	N	N	S	I	G			
81	V	L	G	V	A	P	S	A	S	L	Y	A	V	K	V	L	G	A	D	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	A	N	N	M	D				
	V	L	G	V	S	P	S	A	S	L	Y	A	V	K	V	L	D	S	T	G	S	G	Q	Y	S	W	I	I	N	G	I	E	W	A	I	S	N	N	M	D				
	V	L	G	V	A	P	S	V	S	L	Y	A	V	K	V	L	N	S	S	G	S	G	S	Y	S	G	I	V	S	G	I	E	W	A	T	N	G	M	D					
	V	L	G	V	A	P	S	A	E	L	Y	A	V	K	V	L	G	A	S	G	S	G	S	V	S	S	I	A	Q	G	L	E	W	A	G	N	N	G	M	H				
121	V	I	N	M	S	L	G	G	P	S	G	S	A	A	L	K	A	A	V	D	K	A	V	A	S	G	V	V	V	A	A	A	A	A	A	G	N	E	G	T	S	G		
	V	I	N	M	S	L	G	G	P	T	G	S	T	A	L	K	T	V	V	D	K	A	V	S	S	G	I	V	V	A	A	A	A	A	A	A	G	N	E	G	S	S	G	
	V	I	N	M	S	L	G	G	A	S	G	S	T	A	M	K	Q	A	V	D	N	A	Y	A	R	G	V	V	V	V	A	A	A	A	A	A	G	N	S	G	N	S	G	
	V	A	N	L	S	L	G	S	P	S	P	S	A	T	L	E	Q	A	V	N	S	A	T	S	R	G	V	L	V	V	A	A	A	A	A	A	A	S	G	N	S	G	A	G

SUBSTITUTE SHEET (RULE 26)

FIG.-3A

7/20

161 S S S T V G Y P G K Y P S V I A V G A V D S S N Q R A S F S S V G P E L D V M A  
 S S S T V G Y P A K Y P S T I A V G A V N S S N Q R A S F S S A G S E L D V M A  
 S T N T I G Y P A K Y D S V I A V G A V D S S N Q R A S F S S V G A E L E V M A  
 \* \* \* I S Y P A R Y A N A M A V G A T D Q N N R A S F S Q Y G A G L D I V A

201 P G V S I Q S T L P G N K Y G A Y N G T S M A S P H V A G A A A L I L S K H P N  
 P G V S I Q S T L P G G T Y G A Y N G T S M A T P H V A G A A A L I L S K H P T  
 P G A G V Y S T Y P G S T Y A S L N G T S M A S P H V A G A A A L I L S K H P S  
 P G V N V Q S T Y P G S T Y A S L N G T S M A T P H V A G A A A L V K Q K N P S

241 W T N T Q V R S S L E N T T K L G D S F Y Y G K G L I N V Q A A A Q  
 W T N A Q V R D R L E S T A T Y L G N S F Y Y G K G L I N V Q A A A Q  
 L S A S Q V R N R L S S T A T Y L G S S F Y Y G K G L I N V E A A A Q  
 W S N V Q I R N H L K N T A T S L G S T N L Y G S G L V N A E A A T R

FIG.\_3B

FIG.\_3

FIG.\_3A

FIG.\_3B

8 / 20

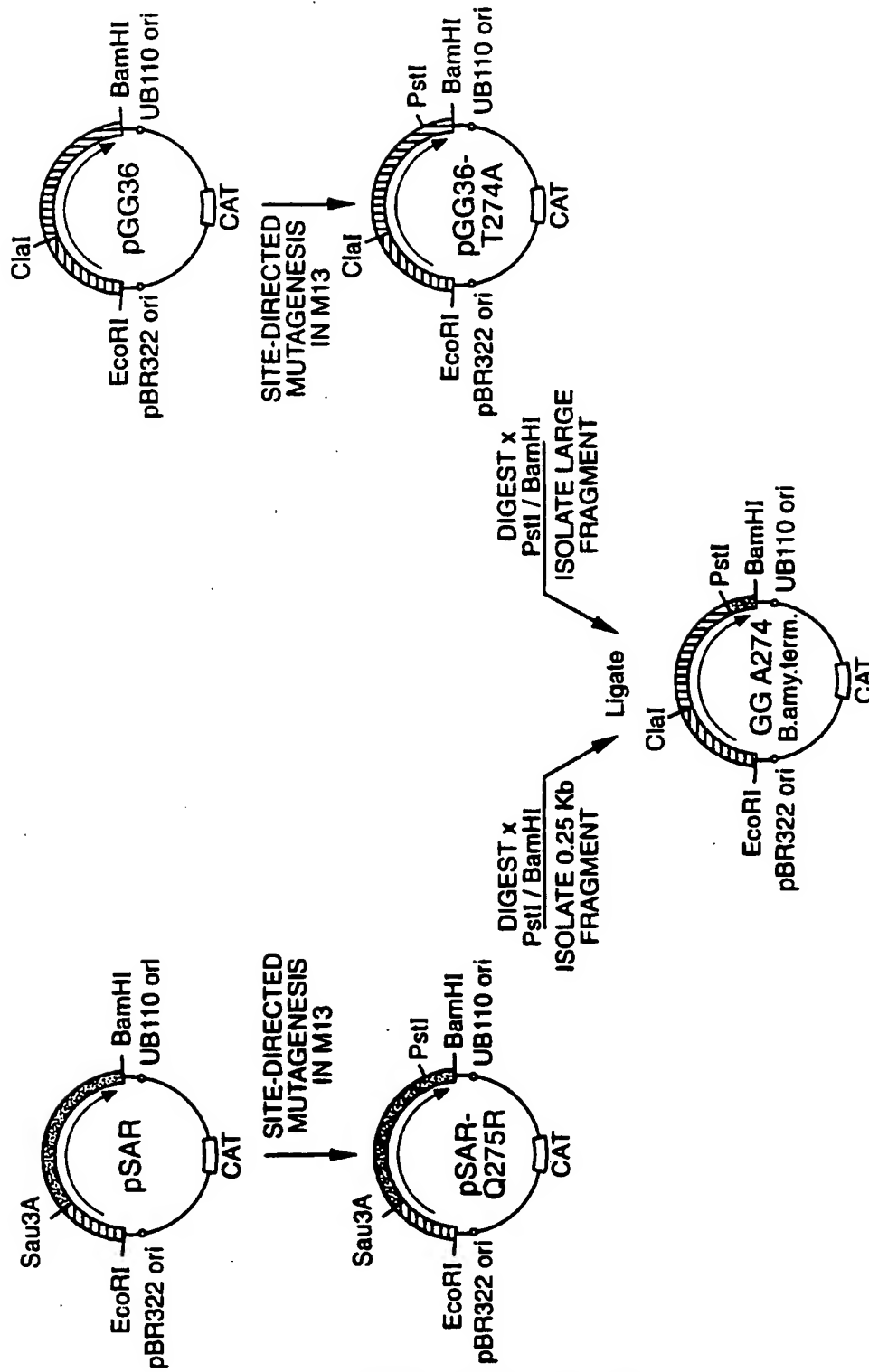
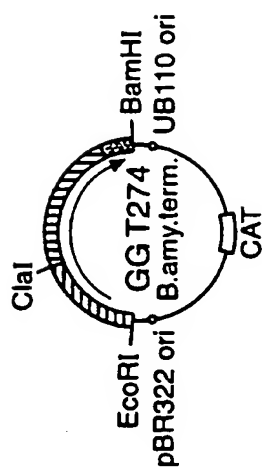
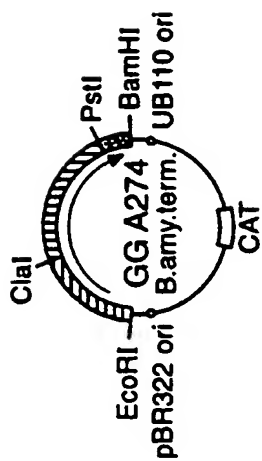


FIG. 4

9 / 20



↑  
SITE-DIRECTED  
MUTAGENESIS  
IN M13

**FIG..5**



10/20

10 30 50  
ATGAAGAAACCGTTGGGGAATAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTT  
MetLysLysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPhe

70 90 110  
AGTTCATCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAAT  
SerSerSerIleAlaSerAlaAlaGluAlaLysGluLysTyrLeuIleGlyPheAsn

130 150 170  
GAGCAGGAAGCTGTCAGTGAGTTTGTAGAACAAAGTAGAGGCAAAATGACGAGTGGCCATT  
GluGlnGluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIle

190 210 230  
CTCTCTGAGGAAGAGGAGTCGAAATTGAATTGCTTTCATGAATTGAAACGATTCCTGTT  
LeuSerGluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProVal

250 270 290  
TTATCCGTTGAGTTAAGCCAGAAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTCT  
LeuSerValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSer

310 330 350  
TATATTGAAGAGGATGCAGAAAGTAACGACAAATGGCGCAATCAGTCCCATGGGGAATTAGC  
TyrIleGluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSer

370 390 410  
CGTGTGCAAGCCCGCTGCCCATAAACCGTGGATTGACAGGTTCTGGTGTAAGAAGTTGCT  
ArgValGlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAla

FIG.\_6A

11/20

430 450 470  
GTCCTCGATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT  
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyAlaSerPhe

490 510 530  
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG  
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590  
ATTGCTGCTTTAAACAATTTCGATTGGCGTTCTTGCCGTAGCCCGGAGCGCGGAACATATAC  
IleAlaAlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650  
GCTGTAAAGTATTAGGGCGAGCGGTTTCAGGTTCCGGTCAGCTCGATTGCCAAGGATTG  
AlaValIleValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeu

670 690 710  
GAATGGGCAGGGAACAATGGCATGCACGTTGCTAATTTCAGTTTAGGAAGCCCTTCGCCCA  
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAlaAsnLeuSerLeuGlySerProSerPro

730 750 770  
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGCGGTTCTTGTGTAGCG  
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830  
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCCGTTATGCCGAACGCAATG  
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

FIG.\_6B

12 / 20

850 870 890  
GCAGTCGGAGCTACTGACCAAAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG  
AlaValGlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly  
910 930 950  
CTTGACATTGTGCGCACCGGTGTAAACGTGCAGAGCACATACCAGGTTCAACGTATGCC  
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla  
970 990 1010  
AGCTTAAACGGTACATCGATCGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAA  
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaLeuValLys  
1030 1050 1070  
CAAAGAACCCTCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAAATACGCAACG  
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr  
1090 1110 1130  
AGCTTAGGAAGCACCGAACTTGATGGAGCGGACTTGTCAAATGCAGAGCGGCAACACGC  
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG.\_6C

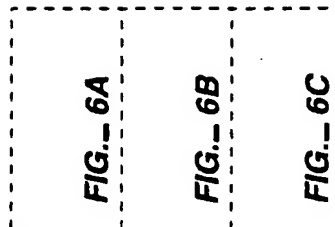


FIG.\_6

**FIG. 7A**

14 / 20

430 450 470  
GTCCTGATACAGGTATTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTT  
ValLeuAspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPhe

490 510 530  
GTACCAGGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCCGGGACG  
ValProGlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThr

550 570 590  
ATTGCTGCTTTAGACAACTCGATTGGCGTTCTTGGCGTAGCCGCCGAGCGGAACTATAC  
IleAlaAlaLeuAspAsnSerIleGlyValLeuGlyValAlaProSerAlaGluLeuTyr

610 630 650  
GCTGTAAAGTATTAGGGCGAGCGGTTTCAGGCGCCATCAGCTCGATTGCCCAAGGATTG  
AlaValIleValLeuGlyAlaSerGlySerGlyAlaIleSerSerIleAlaGlnGlyLeu

670 690 710  
GAATGGGCAGGGAACAATGGCATGCCAGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCA  
GluTrpAlaGlyAsnAsnGlyMetHisValAlaAlaAsnLeuSerLeuGlySerProSerPro

730 750 770  
AGTGCCACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTGTAGCG  
SerAlaThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAla

790 810 830  
GCATCTGGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCCGAACGCAATG  
AlaSerGlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMet

FIG. 7B

15 / 20

850 870 890  
GCAGTCGGAGCTACTGACCAAAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGG  
AlaValGlyAlaThrAspGlnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGly  
910 930 950  
CTTGACATTGTCGCACCGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCC  
LeuAspIleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAla  
970 990 1010  
AGCTTAAACGGTACATCGATGGCTACTCTCATGTTCAGGTGCAGCAGCCCTTGTTAA  
SerLeuAsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaLeuValLys  
1030 1050 1070  
CAAAGAACCCCATCTTGGTCCAAATGTACAAATCCGCAATCATCTAAAGAAATACGGCAACG  
GlnLysAsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThr  
1090 1110 1130  
AGCTTAGGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGC  
SerLeuGlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

FIG..7C

FIG..7A

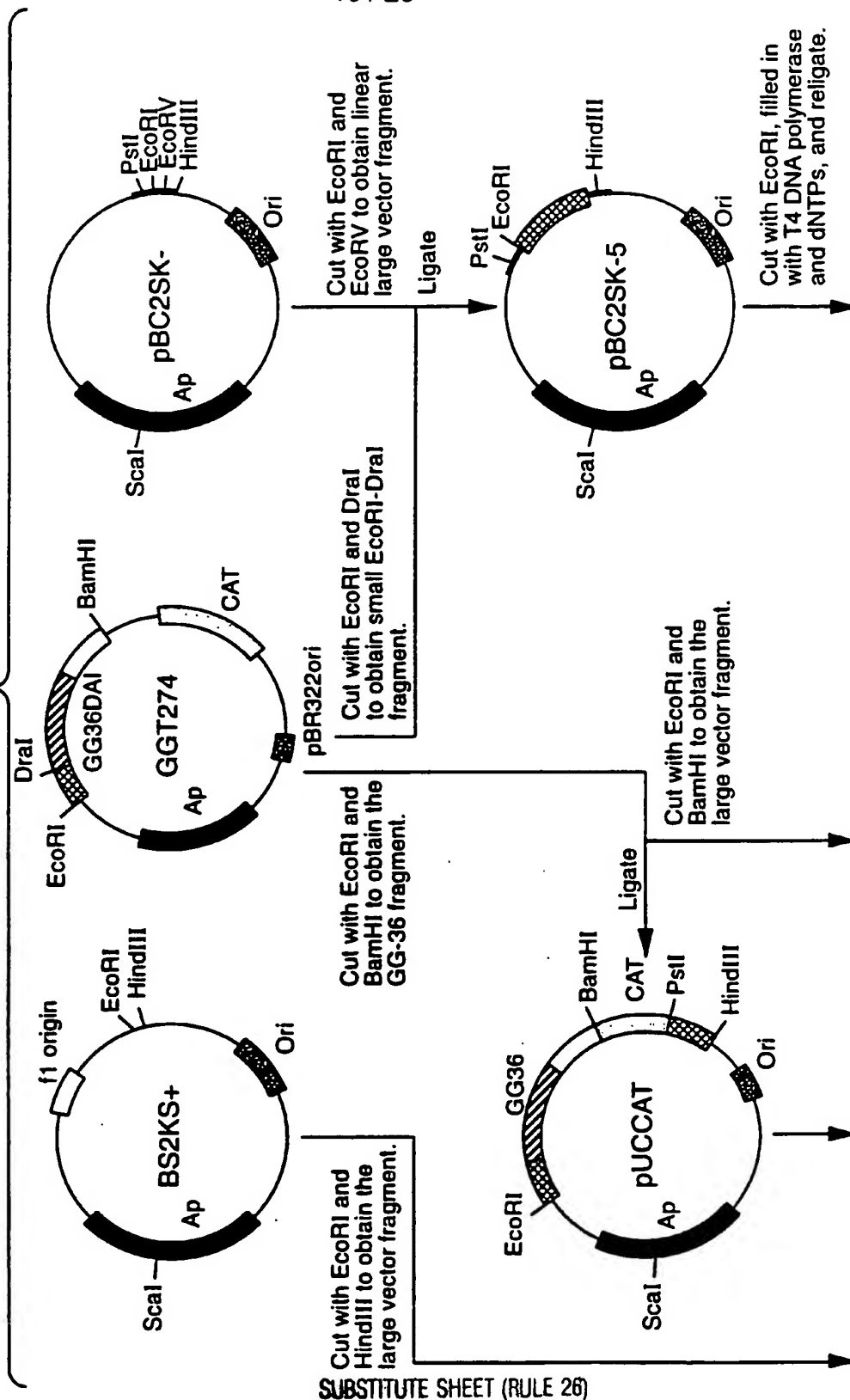
FIG..7B

FIG..7C

FIG..7

16 / 20

**FIG.-8A**



SUBSTITUTE SHEET (RULE 26)

17 / 20

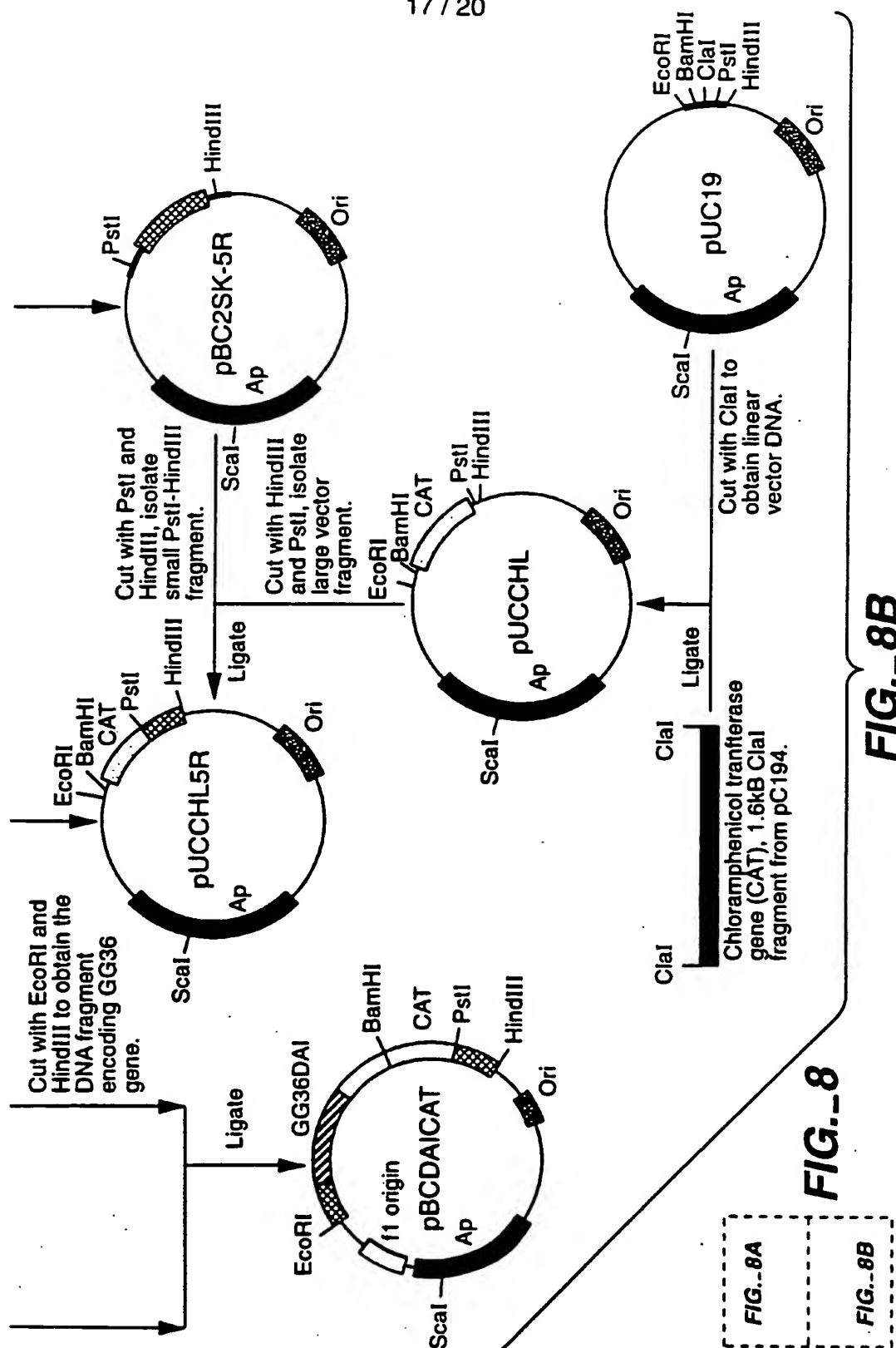


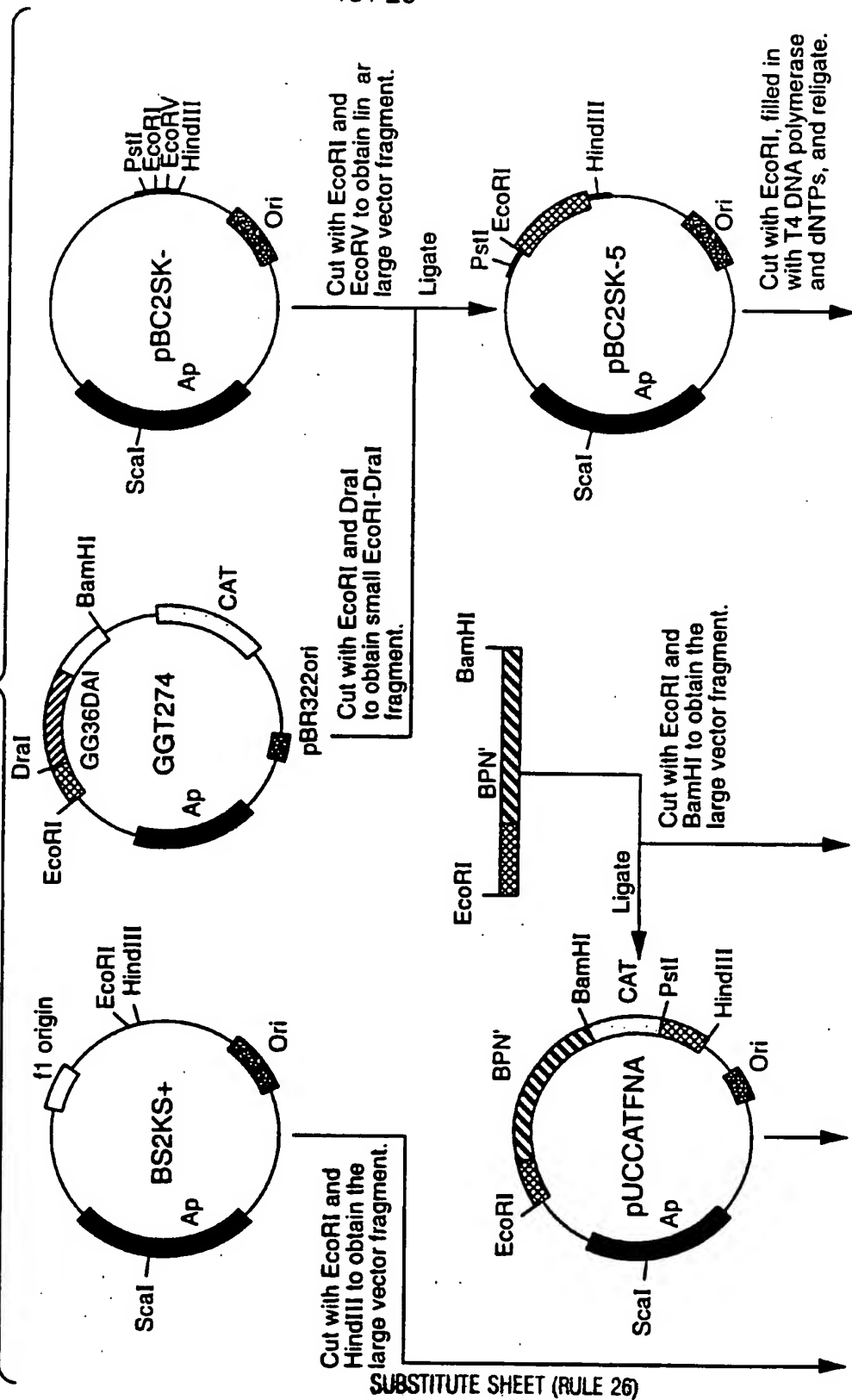
FIG. 8B

FIG. 8



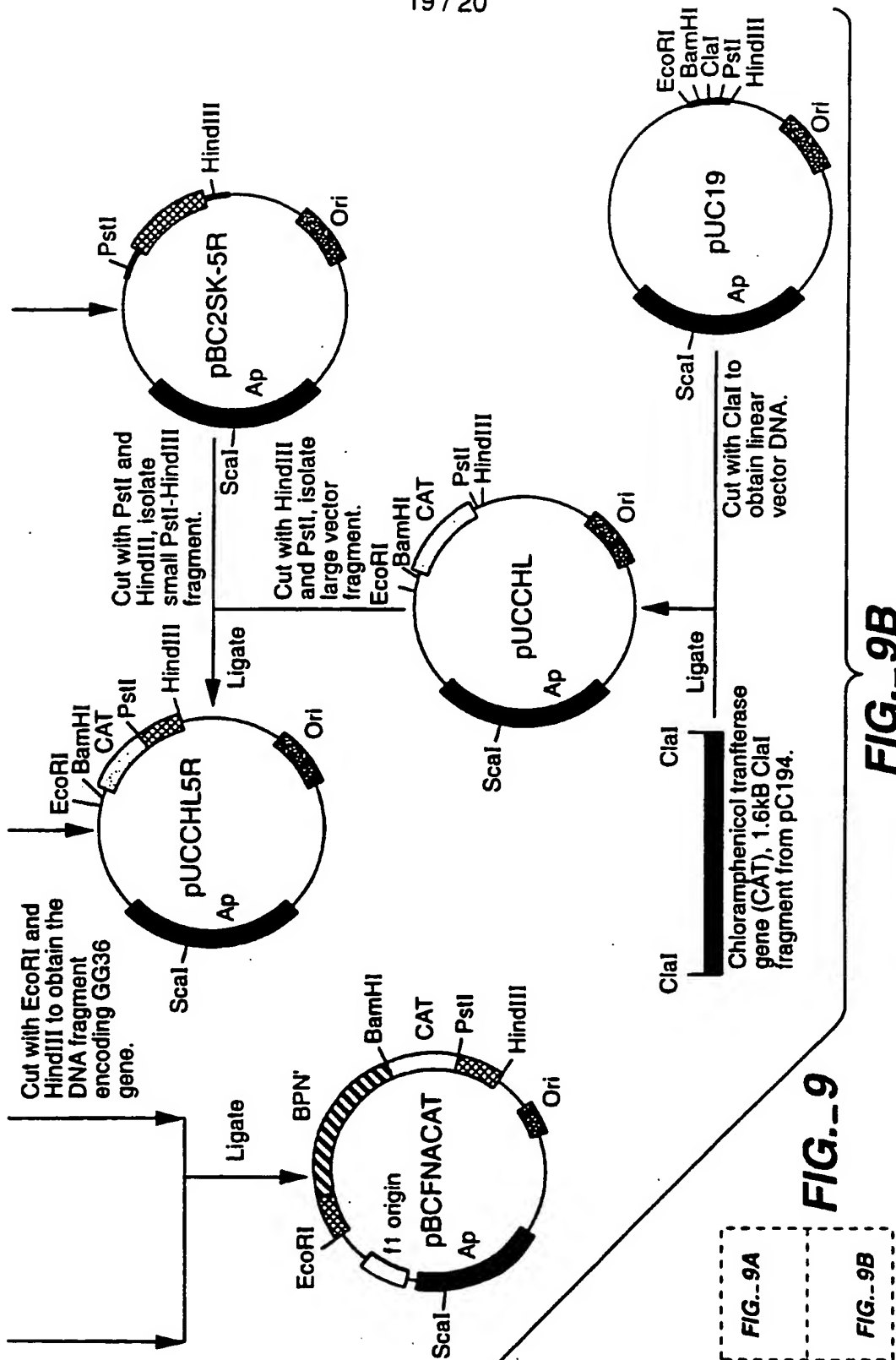
18 / 20

**FIG. 9A**



SUBSTITUTE SHEET (RULE 26)

19 / 20



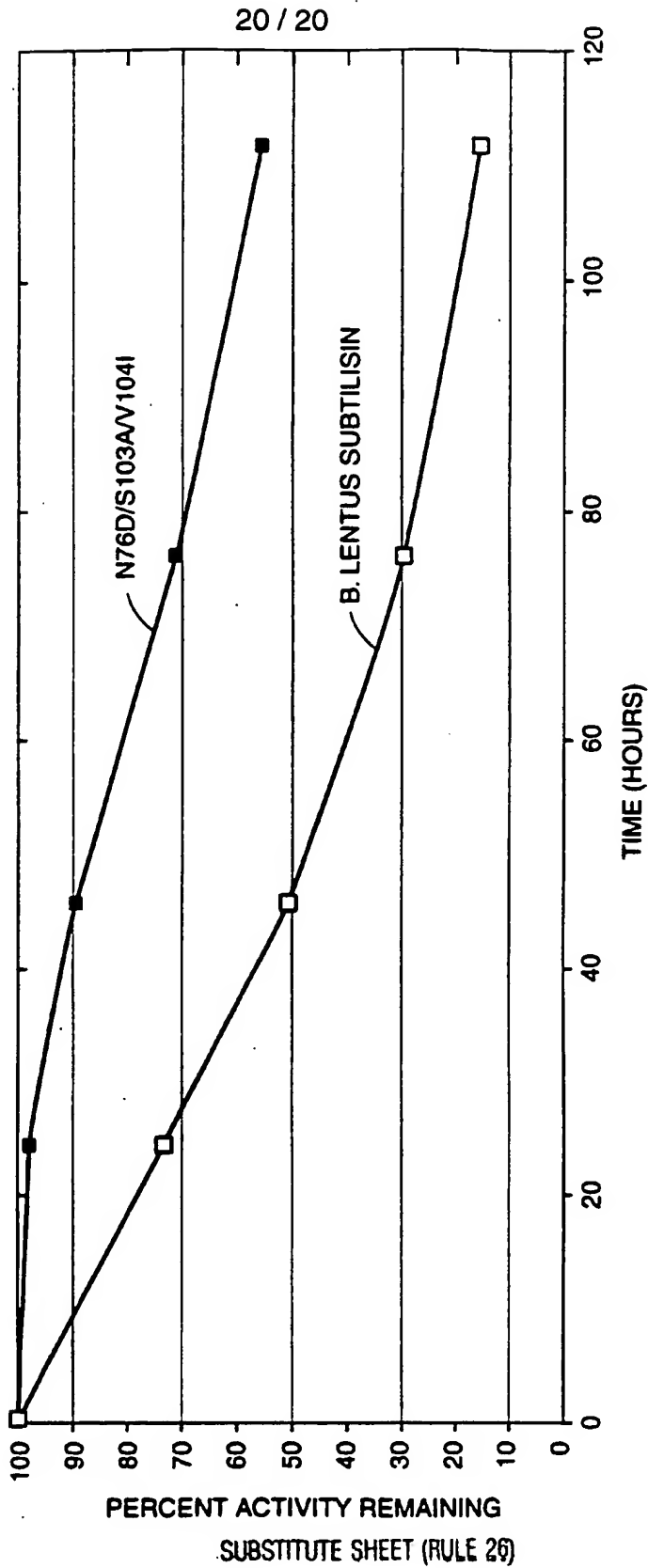


FIG. 10

PCT/US 94/11776

A. CLASSIFICATION OF SUBJECT MATTER  
 IPC 6 C11D3/38 A61K7/48 C12N15/57

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
 IPC 6 C11D C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 398 539 (AMGEN INC.) 22 November 1990 see page 7, line 14 - line 43; claims 1-23; examples ---	1-21
X	EP,A,0 405 901 (UNILEVER) 2 January 1991 see page 30, line 25 - page 35, line 47; claims; examples ---	1-21
X	WO,A,88 08033 (AMGEN INC.) 20 October 1988 see page 14, line 1 - page 15, line 30; claims ---	1-21
X	WO,A,89 09830 (GENEX CORP.) 19 October 1989 see claims 11-16 --- -/--	1-21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

16 February 1995

Date of mailing of the international search report

- 8 03 95

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentaan 2  
 NL - 2280 HV Rijswijk  
 Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl,  
 Fax (+ 31-70) 340-3016

Authorized officer

Grittern, A

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 516 200 (UNILEVER) 2 December 1992 see page 12, line 10 - page 13, line 50; claims 1-10 ---	1-21
A	WO,A,91 06637 (GENENCOR INT.) 16 May 1991 cited in the application see the whole document ---	1
A	WO,A,89 06279 (NOVO INDUSTRI) 13 July 1989 see claims -----	1

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0398539	22-11-90	AU-B- 618675	02-01-92
		AU-A- 5558090	18-12-90
		CA-A- 2016211	17-11-90
		JP-T- 3505976	26-12-91
		WO-A- 9014420	29-11-90
EP-A-0405901	02-01-91	WO-A- 9100334	10-01-91
		JP-T- 4500385	23-01-92
		EP-A- 0405902	02-01-91
		WO-A- 9100335	10-01-91
		JP-T- 4500384	23-01-92
WO-A-8808033	20-10-88	US-A- 4914031	03-04-90
		AU-B- 612653	18-07-91
		AU-A- 1701688	04-11-88
		CA-A- 1316473	20-04-93
		EP-A- 0309565	05-04-89
		JP-T- 1502957	12-10-89
WO-A-8909830	19-10-89	US-A- 5013657	07-05-91
		EP-A- 0409878	30-01-91
		JP-T- 3503602	15-08-91
		US-A- 4990452	05-02-91
		US-A- 5246849	21-09-93
EP-A-0516200	02-12-92	JP-A- 5140588	08-06-93
WO-A-9106637	16-05-91	US-A- 5185258	09-02-93
		AU-B- 633376	28-01-93
		AU-A- 6634190	31-05-91
		CA-A- 2044630	01-05-91
		CN-A- 1052897	10-07-91
		EP-A- 0451244	16-10-91
		JP-T- 4505263	17-09-92
		US-A- 5204015	20-04-93
WO-A-8906279	13-07-89	TR-A- 25991	01-11-93
		EP-A- 0396608	14-11-90
		JP-A- 6292577	21-10-94
		JP-B- 6075504	28-09-94

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-8906279		JP-T- 3503477	08-08-91

**THIS PAGE BLANK (USPTO)**